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Master's project in Life Sciences and Technology

Establishment of polarized microglial cells derived from mouse embryonic stem cells

Carried out in the laboratory of Neural Regeneration
At the Institute of Reconstructive Neurobiology, Bonn
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I Abbreviations

AD	Alzheimer's disease
Arg-1	arginase type 1
ATP	adenosine triphosphate
Aβ	amyloid β
BBB	blood brain barrier
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
CD	cluster of differentiation molecule
CNS	central nervous system
COX-2	cyclooxygenase-2
CXCL10	chemokine (C-X-C motif) ligand 10
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
EB	embryoid body
ESC	embryonic stem cell
ESdM	embryonic stem cell derived microglial precursors
FBS	fetal bovine serum
FGF	fibroblast growth factor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony stimulating factor
h	hours
Iba1	ionized calcium binding adaptor molecule 1
ICAM	intercellular adhesion molecule
ICM	inner cell mass
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
MEF	mouse embryonic fibroblast
MHC	major histocompatibility complex
min	minutes
MS	multiple sclerosis
nGS	normal goat serum
NO	nitric oxide
NOS2	nitric oxide synthase-2
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PD	Parkinson's disease
PFA	paraformaldehyde
PLL	poly-L-lysine
PNS	peripheral nervous system
PRR	pattern recognition receptor
qRT-PCR	quantitative real-time polymerase chain reaction
rm	recombinant mouse
RT	reverse transcription
s	seconds
ScavR1	scavenger receptor type 1
SHP-1	Src homology phosphatase-1
TGF-β	transforming growth factor-β
Th	t-helper cell type
TLR	toll-like receptor
TNF	tumor necrosis factor
TREM2	triggering receptor expressed on myeloid cells 2

II Abstract

Microglia are the resident immune cells of the central nervous system (CNS). Microglia undergo rapid activation in response to even minor pathological changes in the CNS. The activation state of microglia and tissue macrophages is characterized by two extremes that are known as M1 and M2. M1 is triggered upon pro-inflammatory stimuli and lead to the secretion of molecules involved in brain defense but also in neurotoxicity. M2 arises upon anti-inflammatory stimuli and is hallmarked by the secretion of molecules that mediate tolerance and neuroprotection. The aim of this study was to investigate the *in vitro* polarization capacity of microglia into M1 and M2 subtypes. Primary microglia are hard to obtain in sufficient number and therefore a recently developed protocol was applied to differentiate microglial precursors from mouse embryonic stem (ES) cells. The obtained ES cell derived microglial precursors (ESdM) were shown to be an appropriate substitute to primary microglia. Here we showed that ESdM stimulated with interferon-γ (IFN-γ) and lipopolysaccharide (LPS) display typical M1 characteristics like higher transcription levels of pro-inflammatory cytokines as well as an increase in the expression level of M1 surface markers. They also secreted significantly higher levels of the chemokine CXCL10 and nitric oxide than untreated or interleukin-4 (IL-4) treated cells. These results indicate that ESdM can be polarized into a cytotoxic subtype *in vitro* using IFN-γ and LPS. However, no evidence indicating that ESdM were polarized into a neuroprotective M2 subtype by IL-4 treatment has been found. Even though an increase in the transcription level of anti-inflammatory cytokines was observed upon IL-4 treatment, this increase was not significant. In addition, no increase in the expression levels of M2 markers or in the secretion of the anti-inflammatory cytokine IL-10 were found upon IL-4 treatment indicating that IL-4 might be insufficient to polarize ESdM into M2 subtype *in vitro.* Further experiments are required to better understand the mechanisms of microglial sub-differentiation and to determine the stability of the acquired M1 and M2 phenotypes over time.

1. INTRODUCTION

1.1. Microglia

The central nervous system (CNS), composed of the brain and the spinal cord, contains two main cell types: about 100 billion neurons and 10-50 times more supporting cells called glia (Banati, 2003). Neurons are excitable cells that transmit impulses within the CNS and the peripheral nervous system (PNS), which allows the initiation of appropriate responses by the effector tissues in the periphery. Glial cells can be distinguished from neurons through the absence of an axon (Rajadhyaksha and Khan, 2002). They play an essential role in correct neuronal development and in mature neuron functions (Baumann and Pham-Dinh, 2001). Indeed, they maintain homeostasis, form myelin and provide support and protection for neurons. They are therefore known as the "supporting cells" of the nervous system. In the adult brain, the four main functions of glial cells are to surround neurons and hold them in place, to supply nutrients and oxygen to neurons, to insulate one neuron from another and to destroy pathogens and remove dead neurons (Miller *et al.,* 2009). Moreover, they modulate neurotransmission (Auld and Robitaille, 2003). The two major classes of glial cells present in the vertebrate CNS are macroglia, composed mainly of astrocytes and oligodendrocytes, and microglia. It is well accepted that astrocytes and microglia react to CNS damage, and are therefore the two major types of reactive glial cells (Streit *et al.,* 1999; Takuma *et al.,* 2004).

Astrocytes, which are the most prevalent type of glial cells in the CNS (Takuma *et al.,* 2004) have a distinct star-shaped appearance (see Fig. 1.1 A) and perform a large and diverse variety of functions that are essential for the brain. For example, they provide structural, metabolic and trophic support to neurons, control the homeostasis of the neuronal extracellular environment, modulate neuronal synaptic activity and participate in the glial-vascular interface of the blood brain barrier (BBB); (Hatton, 2002; Takuma *et al.,* 2004; Abbott *et al.,* 2006).

Oligodendrocytes (see Fig. 1.1 B) are the myelin-forming cells exclusively in the CNS, as Schwann cells perform this role in the PNS. Processes of the oligodendrocytes contact axons and envelop a stretch of them. Along a single axon, adjacent segments of the myelin sheath belong to distinctive oligodendrocytes. The advantages conferred by these myelin sheaths are the rapid conduction of nerve impulses, fidelity of transfer signaling on long distances and space economy (Baumann and Pham-Dinh, 2001). There are some diseases in which the myelin is affected and therefore the communication between neurons disturbed. This leads to paralysis or motor dysfunction and prove the importance of oligodendrocytes and myelination in the CNS.

Microglia (see Fig. 1.1 C) are the resident immune cells of the CNS and constitute approximately 10-20 % of the total glial cell population. In the CNS they ensure the first line of innate immune response (Raivich *et al.,* 1999; Banati, 2003; Fetler and Amigorena, 2005; Napoli and Neumann, 2009). They were first examined by Pio del Rio-Hortega, who gave them their name in the 1920s. During the 1980s, new staining methods made it possible to visualize them reliably and this almost forgotten glial cell type regained interest. Microglia play important roles throughout life in supporting and maintaining neuronal function, health, homeostasis and survival. Indeed, they phagocytose potentially deleterious debris and secrete neurotrophic factors to promote tissue repair (Streit, 2000; Luo *et al.,* 2010). One of the characteristic features of microglia is that they are quite sensitive to disturbance of the extracellular homeostasis and become rapidly activated in response to even minor pathological changes in the CNS (Kreutzberg, 1996; Streit, 2000). Microglia participate in the initiation of brain inflammation and neuronal death by the production and release of many detrimental factors like pro-inflammatory cytokines, reactive oxygen species and nitric oxide (NO); (Colton, 2009; Henkel *et al.,* 2009). However, it is now well accepted that microglial cells are also able to orchestrate repair and reconstruction in the CNS via the production of anti-inflammatory cytokines and neurotrophic factors (Henkel *et al.,* 2009).

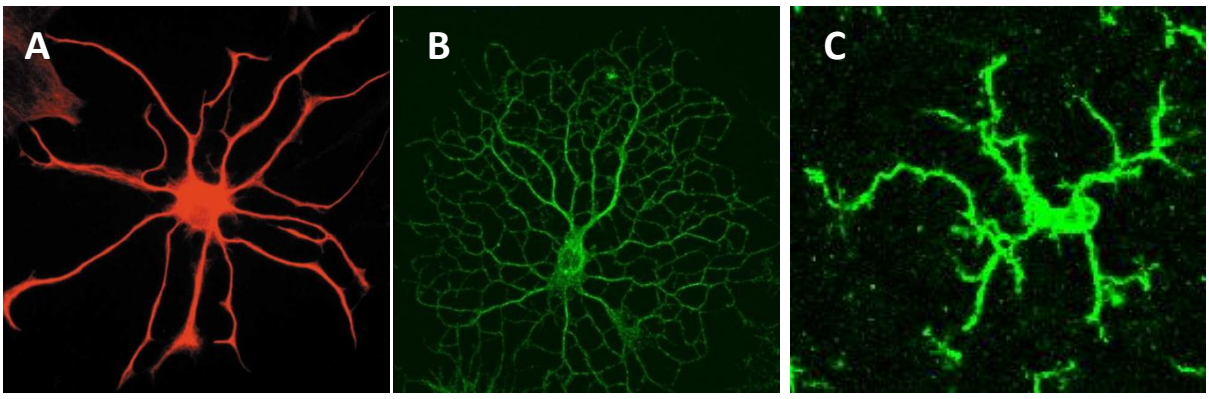

Fig. 1.1: Morphology of the three main glial cells in the brain.
(A) Fluorescent picture of an astrocyte (Picture from P. Magistretti, Institute of Physiology, UNIL, Lausanne). (B) Fluorescent picture of an oligodendrocyte in culture (Picture from Prof. Elek Molnar Lab, Department of Anatomy, University of Bristol). (C) Fluorescent picture of a mouse microglia stained against Iba1 (Picture from L. Bodea, Neural Regeneration Group, Institute of Reconstructive Neurobiology, University of Bonn).

1.1.1. Origin of microglia

Santiago Ramón y Cajal was the first in 1913 who observed a “third element” apart of neurons and neuroglia in the CNS. After that, Pio del Rio-Hortega developed the silver carbonate staining and by using it, was able to identify two different types of cells within this “third element”, the oligodendrocytes and the microglia. These two cell types not only differ by their external morphology but also by their embryonic origin. Indeed, Pio del Rio-Hortega claimed that oligodendrocytes had an ectodermal origin but that microglia were of mesodermal origin (Kaur *et al.,* 2001). Despite intense study, the precise origin and cell lineage of microglia is still under debate, which is mainly due to the lack of cell type-specific markers (Dalmau *et al.,* 2003; Chan *et al.,* 2007). Even if there are actually two main hypotheses concerning their origin, several statements were proposed during the last half-century. The first main hypothesis is that microglial precursors are of neuroectodermal origin like neurons and the remaining glial cells (Fedoroff *et al*., 1997). The second hypothesis states that microglia are derived from mesodermal progenitors and therefore originate from outside the developing nervous tissue. This means that during development these cells have to migrate through extravascular routes, differentiate into microglia and colonize the nervous system (Cuadros and Navascues, 1998; Kaur *et al.,* 2001; Chan *et al.,* 2007).

It was shown that microglia populate the CNS during neuroectodermal development in mice in two waves. First, during embryonic development (E9.5 – E10.5), ionized calcium binding adaptor molecule 1 (Iba1)-positive cells suddenly appear in the brain when the blood circulation has not yet been established (Chan *et al.,* 2007). From E13.5 until around birth, Iba1-positive cells increase in number in the brain and spinal cord (Chan *et al.,* 2007).

During the last decade, it was proposed that two different types of microglia are present in the brain: microglia produced during development and the ones that are responsible for microglial turnover in the adult brain and under pathological conditions (Cuadros and Navascues, 1998). The first type of microglia populates the nervous system primarily during embryonic and fetal development (Cuadros and Navascues, 1998). The second type are microglia derived from bone-marrow that are able to migrate into the adult CNS under certain specific pathological conditions (Ladeby *et al.,* 2005; Simard *et al.,* 2006; Mildner *et al.,* 2007). However, it was shown that microgliosis in the adult brain is mainly due to the proliferation of CNS resident microglia (Ransohoff, 2007).

1.1.2. Microglial morphologies and their functional roles

Microglial cells are extremely plastic cells and undergo a variety of structural changes depending on their location and environment. Three distinct forms with different functions can be observed in the CNS depending on the brain state; amoeboid, resting/ramified and reactive/activated microglia.

Amoeboid microglia

Amoeboid microglial cells are mainly associated with the developing CNS (Ling and Wong, 1993; Dalmau *et al.,* 1997; Ladeby *et al.,* 2005). These microglial cells exhibit a large round cell body with few filopodia, which allows them to move easily throughout the neural tissue where they fulfill their scavenger role. Amoeboid microglia are able to phagocytose debris, but they do not accomplish the same antigen-presenting and inflammatory roles as activated microglia (Brockhaus *et al.,* 1996; Cuadros and Navascues, 1998). Once they arrive at their final destination, the vast majority of amoeboid microglia differentiates into primitive ramified microglia, while few undergo apoptosis (Dalmau *et al.,* 2003; Ladeby *et al.,* 2005). Primitive ramified microglia constitute an intermediate state between amoeboid and ramified microglia. They are poorly ramified and are found in the brain during the period of enormous microglial proliferation (Dalmau *et al.,* 2003; Ladeby *et al.,* 2005).

Resting / ramified microglia in the normal brain

Ramified microglia (see Fig. 1.2 A), which form 10-20 % of the total glial population (Raivich *et al.,* 1999) are found in the intact brain. Their distribution is rather homogeneous but non-overlapping, and they show a slow turnover. Their state is often referred to as “resting” (Luo *et al.,* 2010) but it was shown that resting microglia actively screen the entire CNS parenchyma with their highly motile processes every few hours (h); (Fetler and Amigorena, 2005). Unlike the amoeboid form of microglia, the somata of the ramified microglia generally remain fixed (Nimmerjahn *et al.,* 2005). Their processes were observed to directly contact astrocytes, neuronal cell bodies, neuronal synapses and blood vessels suggesting that in healthy brain, microglia dynamically act together with other cortical cells (Nimmerjahn *et al.,* 2005; Wake *et al.,* 2009). Unlike amoeboid or activated microglia, resting microglia have no phagocytic activity (Streit *et al.,* 1999) and they express low levels of major histocompatibility complex (MHC) antigens and activation markers (Tsuchiya *et al.,* 2005). They therefore display very low antigen-presentation capacity. However, resting microglia constitutively express a number of surface molecules including cluster of differentiation molecule (CD)11b, CD68, F4/80 and Iba1, which are often used to identify microglia (Guillemin and Brew, 2004).

Therefore, it is likely that ramified microglia monitor the well-being of the brain cells and also clean the extracellular fluid in order to maintain central homeostasis and an immunologically silent environment (Booth and Thomas, 1991; Thomas, 1992; Fetler and Amigorena, 2005).

Reactive / activated microglia in the injured brain

In case of trauma or pathogen invasion as well as inflammatory and chronic neurodegenerative diseases, resting microglia become gradually activated and undergo morphological as well as functional changes (Raivich *et al.,* 1999; Ransohoff, 2007). Indeed, they rapidly switch from their ramified shape to a rounded (rod-shape) morphology (see Fig. 1.2 B and C) and 2-3 days after injury, their number is increased through mitotic division (Streit *et al.,* 1999; Ladeby *et al.,* 2005). Although microglia number predominantly increases due to resident microglia proliferation, a process called reactive microgliosis, it was shown that these cells are supplemented by microglia from adjacent brain areas as well as by recruited bone marrow-derived cells (Ladeby *et al.,* 2005; Simard *et al.,* 2006; Mildner *et al,* 2007). Upon neuronal death, microglia might acquire a macrophage-like morphology with phagocytic activity (see Fig. 1.2 C; Kreutzberg, 1996; Raivich *et al.,* 1999). Activated microglial cells migrate into the lesion site, surround damaged or dead cells, clear cellular debris by phagocytosis and release inflammatory cytokines (Tanaka *et al.,* 2003; Fetler and Amigorena, 2005; Napoli and Neumann, 2009). At the molecular level, there is an up-regulation or *de novo* expression of different cell surface receptors, cytokines, growth factors, free radicals and NO (Streit, 2000; Mantovani *et al.,* 2004; Ladeby *et al.,* 2005). Moreover, microglial activation may result in expression of MHC antigens and costimulatory molecules (Hoftberger *et al.,* 2004; Ladeby *et al.,* 2005; Tsuchiya *et al.,* 2005).

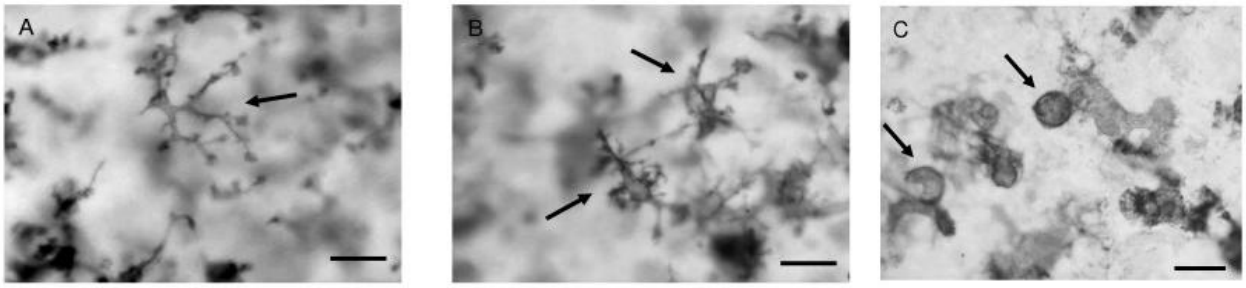

Fig. 1.2: Progressive change in microglial morphology upon activation.
Rat hippocampus slice stained with OX-42 (recognizes the rat counterpart of the microglial cell surface antigen CD11b and CD11c) at 8 days *in vitro* after 24 h exposure to 5 µg/ml LPS. All shapes of microglia were found within the slice. (A) "Resting", ramified microglia, (B) "reactive", activated microglia, and (C) "phagocytic", macrophage microglia. Scale bars: 50 µm (from Huuskonen *et al.,* 2005).

1.1.3. Characterization of microglia using surface markers

The characterization of microglia using surface expression markers is difficult, as they share most markers with macrophages. However, studies define the following surface marker profile for microglia: $CD68^{+}$, $CD45^{low}$, $CD11b^{+}$, $CD11c^{high}$, MHC class II^{+}, $Iba1^{+}$ and $F4/80^{+}$ (Guillemin and Brew, 2004). Iba1 is probably the most versatile immunocytochemistry marker for microglial cells (Graeber and Streit, 2010).

1.1.4. Microglial activation

Microglial cells are responsible for the first line of immune defense in the CNS. The activation of microglia is a graded response to a variety of signals or stimuli (see Fig. 1.3; Raivich *et al.,* 1999; van Rossum and Hanisch, 2004). Microglia become activated under even minor changes in the CNS, including the presence of pathogens, pathogen components and infectious diseases of the CNS as well as acute or chronic neurodegenerative diseases (Raivich *et al.,* 1999; Ransohoff, 2007).

Pathogens are detected by microglia by the means of pattern recognition receptors (PRRs) located on their cell surface (Colton, 2009). These PRRs recognize the so-called pathogen-associated molecular patterns (PAMPs), which are found only on the cell surface of pathogens. Most PRRs are only expressed at low levels on ramified microglia and are up-regulated upon microglial activation (Aloisi, 2001).

The most common activation compound used *in vitro* to mimic gram-negative bacterial infections is the bacterial endotoxin lipopolysaccharide (LPS); (Hanisch, 2002). LPS induces an inflammatory reaction in microglia, leading to the production of pro-inflammatory cytokines and chemokines (Nakajima *et al.,* 2003), tumor necrosis factor (TNF)-α and nitric oxide synthase-2 (NOS2) (Napoli and Neumann, 2009). The Toll-like receptor (TLR)-4 was shown to be a central mediator of the LPS induced activation of microglia (Kalis *et al.,* 2003).

Local tissue damage is also a stimulus able to activate microglia, suggesting that the damage cells produce a signal that activates microglia. Indeed, it is known that nucleotides such as adenosine triphosphate (ATP) are released by injured neurons and attract microglia via activation of purinoceptors (Honda *et al.,* 2001; Haynes *et al.,* 2006). In the presence of apoptotic cells, microglia can phagocytose them without triggering an inflammation. Indeed, they release anti-inflammatory cytokines such as transforming growth factor-β (TGF-β); (Napoli and Neumann, 2009).

Activated microglia share many functions with tissue macrophages, including phagocytosis and antigen presentation (in association with MHC class II molecules) to circulating T-cells. They also produce a lot of inflammatory cytokines (see Fig. 1.3) like interleukin (IL)-1, IL-6, IL-10, chemokine (C-X-C motif) ligand 10 (CXCL10) and TNF-α, chemokines, complement components, reactive oxygen intermediates and NO (Liu *et al.,* 1998; Gregersen *et al.,* 2000; Hanisch, 2002; Mack *et al.,* 2003; Mantovani *et al.,* 2004). It is important to note that microglial activation is a transient and self-limited process and usually declines within about one month after injury, even after severe traumatic and/or ischemic insults (Streit *et al.,* 1999).

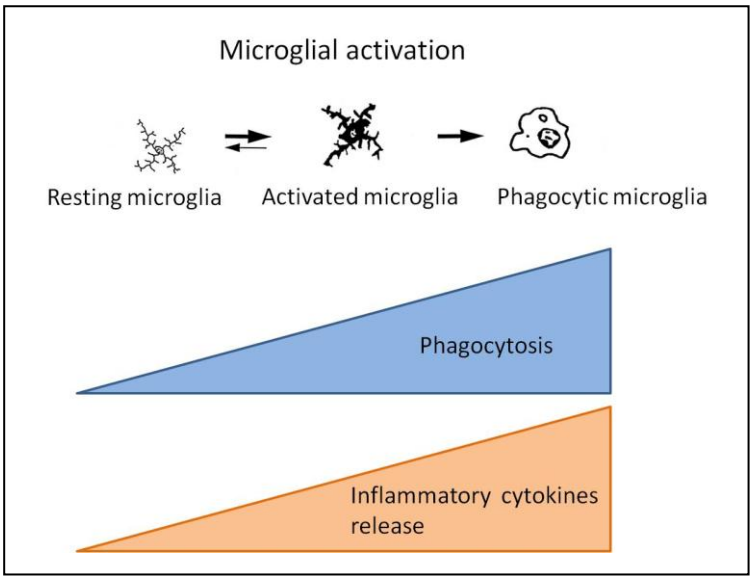

Fig. 1.3: Microglial activation in response to pathological events such as disease or injury. Morphology of microglia progressively changes from a ramified to an amoeboid form depending on the degree of the injury. In the course of microglial activation, phagocytosis capacity of microglia is enhanced. Upon activation, pro-inflammatory molecules are released (modified from Streit, 1999; Kettenmann, 2007).

Albeit the absence of injury or damage, microglia become progressively more activated with age. Indeed, microglial hypertrophy and rod cell formation can be observed in aged brains. Moreover, microglia start to fuse together, forming clusters of cells that are thought to form progressively larger aggregates eventually culminating in the formation of senile plaques (Streit and Sparks; 1997; Luo *et al.,* 2010).

Inflammatory mediators

The main communication mode between microglia and other cells of the immune system is the synthesis of many different cytokines, which is an inducible process upon request (Hanisch, 2002). Cytokines may mediate the recruitment of other microglia and peripheral immune cells and affect the activation state of microglia (Hanisch, 2002). Mainly, cytokines are grouped into pro- and anti-

inflammatory mediators. Microglial activation can induce the initial release of both cytokines types, and some cytokines can have an autocrine impact on further release activity (Hanisch, 2002). Essential cytokine systems for microglia include members of IL family, IFNs, TNF and relatives, and chemokines (Hanisch, 2002). Different stimuli induce the expression of distinctive cytokines in the CNS. To give an example, indirect trauma such as in the facial axotomy model, leads to the expression of IL-6 followed by that of TGF-β1 (Raivich *et al.,* 1999). Direct brain injury, convulsion and Alzheimer's disease (AD) typically lead to the additional induction of IL-1β and TNF-α. Infectious inflammation due to viral and bacterial meningitis and encephalitis, human immunodeficiency virus, malaria and autoimmune diseases, cause the additional synthesis of IFN-γ (Raivich *et al.,* 1999). To summarize, the expression pattern of cytokine can be illustrated by a pyramid, as increased brain pathology leads to a step by step enrollment of more and more pro-inflammatory cytokines (Raivich *et al.,* 1999).

ILs are a group of cytokines which are key signaling molecules for many immune cells including microglia. IL-1 is a pro-inflammatory cytokine that exists in two isoforms, IL-1α being mostly cell-associated and IL-1β being the major soluble form. IL-1 is a crucial microglial effector cytokine and plays central role in innate defense and immune response (Hanisch, 2002). IL-1 is released during inflammation by activated microglia and invading immune cells. *In vitro*, it was shown that IL-1 induces expression of the pro-inflammatory cytokines TNF-α and IL-6 as well as the enzyme cyclooxygenase-2 (COX-2) in both astrocytes and microglia (Shaftel *et al.,* 2008).

IL-4, IL-10, IL-13 and TGF-β have anti-inflammatory, immunosuppressive and neuroprotective effects, which can be attributed to a down-regulation of pro-inflammatory cytokines production by glial cells or to the attenuation of their secondary release actions (Hanisch, 2002). A self-regulating mechanism may therefore exist, with the potential to control inflammation.

IFNs are a group of widely expressed cytokines that affect cell growth, immunomodulation and communication between cells. They play a major role in the first line of defense against viral infections (Hanisch, 2002). Two main types of IFNs exist: type I and type II IFNs. IFN-α and IFN-β are two kinds of type I IFN and are released upon viral infection (Hanisch, 2002). IFN-γ is the only type II IFN and is produced by certain activated T-cells and natural killer cells. In microglia, IFN-γ causes induction and up-regulation of many different cell surface molecules; MHC class I and II, intercellular adhesion molecule (ICAM)-I, immune-accessory molecules B7 (CD80/86), leukocyte function-associated molecule 1, LPS receptor (CD14), Fc and complement receptors. It also induces changes in the proteasome composition, as well as release of cytokines (TNF-α, IL-1, IL-6), components of the complement system (C1q, C2, C3, C4), and NO (Hanisch, 2002).

1.1.5. Microglial sub-differentiation

The absence of neurogenesis, the limited regeneration of injured nerves and the vulnerability to degenerative conditions has long been considered as the cause of poor recovery from acute insults or chronic degenerative disorders in the CNS (Butovsky *et al.,* 2006). However, during the last decade, it has been shown that the brain undergoes neurogenesis throughout life to a limited extent (Kuhn *et al.,* 1996; Eriksson *et al.,* 1998, Ekdahl *et al.,* 2009). Brain inflammation, which activates microglia, strongly attenuates neurogenesis and might play an important role in the pathogenesis of chronic neurodegenerative disorders like AD and Parkinson's disease (PD); (Ekdahl *et al.,* 2003). More recent studies have shown, however, that a local and well controlled immune response can be beneficial, support survival and promote recovery in the brain, whereas an uncontrolled one impairs neuronal survival and repair processes and may lead to autoimmune disease (Butovsky *et al.,* 2005). During an inflammatory reaction, the pro-inflammatory process is important to permit the elimination of causative infectious, toxic or allergenic agents. However, it is important that this pro-inflammatory process, once induced, does not escalate, but is down-regulated to allow healing (Gratchev *et al.,* 2001). Therefore pro- and anti-inflammatory processes have to be activated spatially and temporally in a very precise manner (Goerdt *et al.,* 1999; Goerdt and Orfanos, 1999). Microglia activation, which is an indication of brain inflammation, is therefore no more seen as being either toxic or protective for neurons. The consequences of their activation depend on the balance between the secretion of anti- and pro-inflammatory molecules.

Microglia might be sub-differentiated *in vitro* in two different subtypes, type I (M1) microglia and type II (M2) microglia which represent the extremes of the differentiation spectrum (see Fig. 1.4). These two types of microglia differ in terms of receptor expression, cytokine production, effector function and cytokine repertoires (see Fig. 1.4; Mantovani *et al.,* 2004). The M1 subtype is similar to that of the classically activated pro-inflammatory microglia whereas the M2 subtype corresponds to the alternatively activated anti-inflammatory microglia (Stein *et al.,* 1992; Michelucci *et al.,* 2009, Kigerl *et al.,* 2009). The M1 subtype are triggered to respond to microbial compounds like LPS or pro-inflammatory cytokines like IFN-γ and T helper cell type (Th) 1-derived lymphokine (Mantovani *et al.,* 2004; Michelucci *et al.,* 2009). Their activation induces the production of pro-inflammatory cytokines such as IL-1, IL-12 and TNF-α, free radicals like NO and superoxide anions (Goerdt and Orfanos, 1999). Although these molecules are essential for the defense of the brain, their expression also causes a lot of collateral damage to the cells and tissues (Ding *et al.,* 1988).

Microglia might be activated into the M2 subtype in the presence of a variety of Th2 lymphocyte products such as IL-4, IL-10, IL-13 and TGF-β (Kigerl *et al.,* 2009; Goerdt and Orfanos, 1999). They have anti-inflammatory properties and preferentially express receptors involved in endocytosis or phagocytosis such as macrophage mannose receptor and scavenger receptor type 1 (ScavR1) (Stein *et al.,* 1992; Goerdt and Orfanos, 1999). They are important to mediate tolerance, to downregulate inflammation and to protect the tissues against oxidative damage (Goerdt *et al.,* 1999; Ghassabeh *et al.,* 2006). It was recently shown that they have improved clearance function of oligomeric amyloid-β (Aβ) (Shimizu *et al.,* 2008).

These two types of microglial cells permit to balance pro- and anti-inflammatory immune reactions in the brain. Recent *in vitro* studies have shown that microglia activated by IFN-γ (M1) prevent oligodendrogenesis and neurogenesis whereas microglia activated by IL-4 (M2) induced both (Butovsky *et al.,* 2006). Moreover, Kigerl and colleagues have claimed that the polarization of resident macrophages and infiltrating blood monocytes into M2 subtype could limit secondary inflammatory-mediated injury and therefore promote CNS repair (Kigerl *et al.,* 2009).

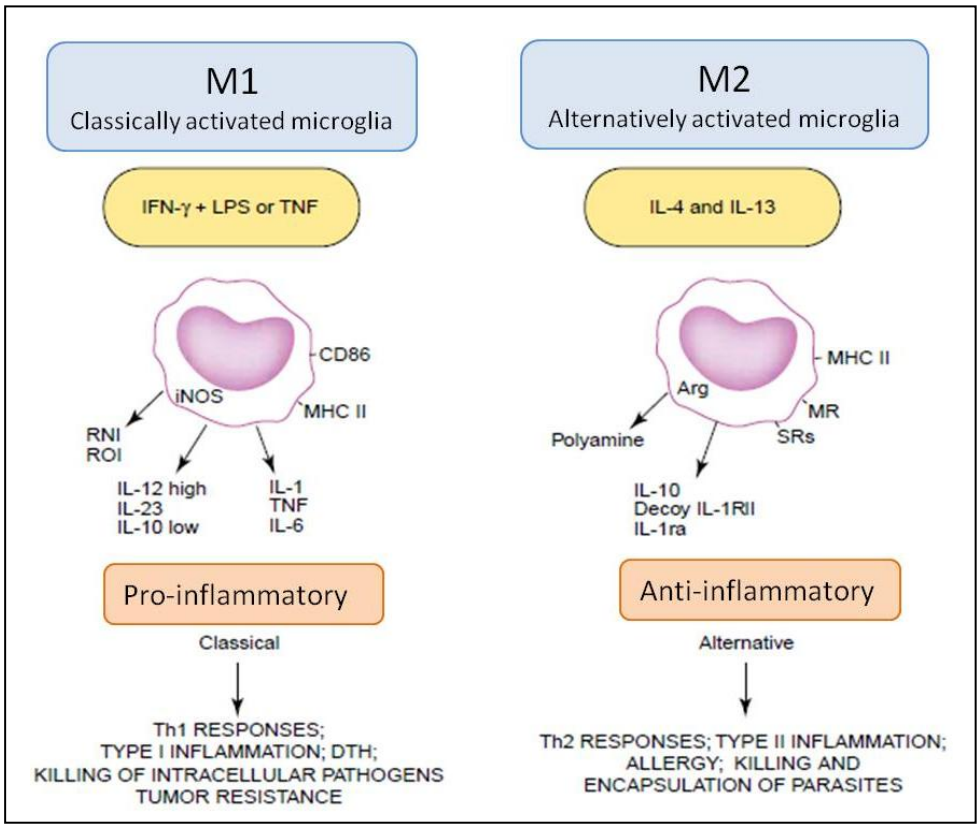

Fig. 1.4: Overview of microglial sub-differentiation into M1 or M2 subtype.

Microglia polarize and acquire different functional properties in response to environment derived signals. IFN-γ and LPS drive M1 polarization, with potentiated cytotoxic and anti-tumoral properties whereas M2 microglia drive Th2 response.

Abbreviations: DTH, delayed-type hypersensitivity; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MR, mannose receptor; RNI, reactive nitrogen intermediates; ROI, reactive oxygen intermediates; SRs, scavenger receptors (modified from Mantovani *et al.*, 2004).

1.2. Embryonic stem cells

An embryo is formed and develops through a process called embryogenesis. The fertilization of the egg gives rise to a zygote, which possesses the ability to generate the entire organism. This capacity is known as totipotency and is retained up to the eight-cell stage of morula (Wobus and Boheler, 2005). Subsequent cell differentiation gives rise to the formation of the blastocyst composed of the inner cell mass (ICM); (or embryoblast) which forms the embryo, and the outer layer of cells (or trophoblast), which later forms the placenta. The cells of inner cell mass are pluripotent as they retain the ability to form all the cell types of the embryo (Wobus and Boheler, 2005).

In 1981, the group of Evans and Kaufman succeeded in cultivating mouse pluripotent stem cells from blastocysts (see Fig. 1.5; Evans and Kaufman, 1981). These cells, called embryonic stem cells (ESC), originated from the ICM and could be maintained *in vitro* on a feeder layer of inactivated mouse embryonic fibroblasts (MEF) without losing their differentiation potential. To prove the pluripotency of these cells, mouse chimeras were created by injection into blastocysts and it was shown that ESC could contribute to all cell lineages including the germ line (Bradley *et al.,* 1984). *In vitro*, murine ESC lines have an almost unlimited proliferation capacity and retain the ability to contribute to all cell lineages. Moreover, they conserve a relatively normal and stable karyotype, even during further passaging (Evans and Kaufman, 1981; Smith, 2001).

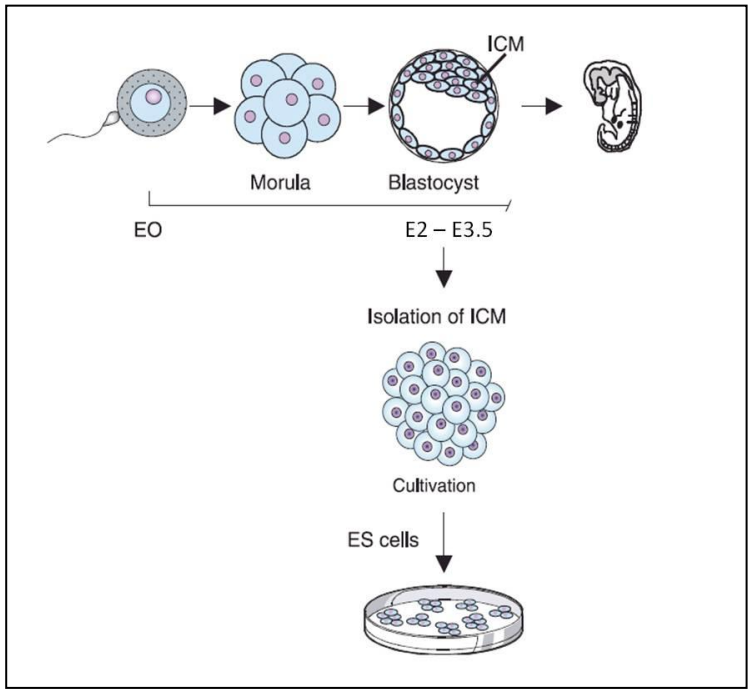

Fig. 1.5: Developmental origin of pluripotent embryonic stem cell lines of the mouse.
The scheme demonstrates the derivation of embryonic stem cells between embryonic day E2 to E3.5. ESCs are derived from the ICM of blastocysts (Modified from Wobus and Boheler, 2005).

The inactivated MEF layer is used because it supplies important factors either to promote self-renewal or to inhibit differentiation of ESCs (Wobus and Boheler, 2005). For example, it was shown that leukemia inhibitory factor (LIF), a soluble glycoprotein of the IL-6 class cytokine has both effects on ESCs (Williams *et al.,* 1988). It was shown that LIF supplementation to the culture media has little effect on growth rates, but it significantly altered the probability of cells undergoing self-renewal versus differentiation (Zandstra *et al.,* 2000).

1.2.1. *In vitro* differentiation potential of ESCs

During mouse embryogenesis, the ICM proliferates rapidly and gives rise to the primitive ectoderm from which three germ layers are derived: endoderm, mesoderm and ectoderm. These three germ layers give rise to all tissue and organs of the developing embryo (Wobus and Boheler, 2005).

Differentiation of ESCs *in vitro* is induced by triggering embryoid body (EB) formation in the absence of self-renewal signals provided by feeder cell layers or LIF (Wobus and Boheler, 2005). Once differentiation has begun, spontaneous development of cells representing the three primary germ layers inside the EBs occurs. These EBs are then transferred to culture tissue dishes to allow continued differentiation into a range of specialized cell types including cardiac, smooth and skeletal muscle as well as hematopoietic, pancreatic, hepatic, lipid, cartilage, or neuronal and glial cells (Wobus and Boheler, 2005). A carefully chosen combination of signal proteins can be used to induce the differentiation of the desired cell type.

1.3. Embryonic stem cell derived microglial precursors (ESdM)

Microglia were shown to be major players in some neurodegenerative diseases like AD, PD and multiple sclerosis (MS) (Streit, 2004). Therefore, the idea to use them in therapy approaches for such diseases is pretty attractive. One major problem is the difficulty to obtain sufficient amounts of primary microglia by traditional procedures for biochemical analysis and *in vivo* experiments (Napoli *et al.,* 2009). Classical procedures to obtain primary microglia are either the isolation of microglia from mixed glial cultures derived from early mouse brain tissue or the isolation of microglia directly by flow cytometry (Ford *et al.,* 1995; Havenith *et al.,* 1998). As these procedures are very time consuming, many researchers have used oncogenically transformed microglial cell lines like BV-2 or N9 instead of primary microglia (Blasi *et al.,* 1990; Bocchini *et al.,* 1992). The problem with these cell lines is that the original microglial characteristics and functions might be affected due to the oncogenical transformations (Horvath *et al.,* 2008).

To avoid all these problems, Napoli and colleagues have developed a protocol to differentiate microglia from mouse ESCs (Napoli *et al.,* 2009; Beutner *et al.,* 2010) by modification of a protocol for neuronal differentiation (Lee *et al.,* 2000). This protocol provides a tool to obtain unlimited numbers of ESdM without any oncogenic modification. The protocol contains five main steps; (1) culture of ESCs on a MEF layer in LIF containing medium, (2) formation of EBs in non adherent dishes followed by transfer into adherent dishes for EBs differentiation, (3) selection of neuronal precursor cells, (4) expansion of these neuronal precursor cells and (5) differentiation into microglial precursor cells. In total, approximately seven weeks are necessary to obtain ESdM.

It was shown that ESdM display on average a doubling time of about 20 h. ESdM express microglial markers like Iba1, CD11b, CD45, CD86 (B7.2), α4 and β1 integrins and F4/80, but they are negative for stem cell markers CD117 (cKit) and CD34. Moreover, and like primary microglia, ESdM display little or very low constitutive expression of MHC class II. The profile of cytokines expression of ESdM resembles that of primary microglia and BV-2 after LPS and IFN-γ stimulation. ESdM express the receptor CX3CR1 and are able to migrate toward the chemokines CX3CL1 in a way similar to primary microglia. Upon stimulation with LPS, ESdM are able to phagocyte microsphere beads, which is an important functional aspect of microglia. Moreover, after *in vivo* transplantation, ESdM engraft next to injection sides within the parenchyma of the cortex and hippocampus (Napoli *et al.,* 2009; Beutner *et al.,* 2010). RNA microarray of 44 000 genes was also performed to compare the gene expression profile of primary microglia to ESdM. 20 000 gens were found to be expressed in primary microglia and ESdM. Of these expressed genes, over 19 000 were co-expressed by primary microglia and ESdM, meaning that ESdM have a similar genetic profile as primary microglia (data obtained by Clara Beutner, unpublished).

Thus, the differentiation of ESCs into ESdM is an ideal tool to obtain sufficient cells number for biochemical analysis and *in vivo* experiments. Moreover, ESdM are an adequate substitute for primary microglia as they display properties alike of primary microglia.

1.4. Aim of the study

One major problem one has to face when working with microglia is the difficulty to obtain sufficient amounts of primary microglia by isolation and enrichment from mixed glial cultures derived from early mouse brain tissue or by isolation of microglia directly by flow cytometry (Ford et al., 1995; Havenith et al., 1998) for biochemical analysis and *in vivo* experiments. Napoli and colleagues developed a protocol to differentiate microglia from mouse ESCs (Napoli *et al.,* 2009; Beutner *et al.,* 2010). ESdM were shown to be an appropriate substitute to primary microglia (Napoli *et al.,* 2009; Beutner *et al.,* 2010) and using this protocol, it is possible to obtain a high number of ESdM and therefore, biochemical analysis and *in vivo* experiments are possible.

Microglia can have both, cytotoxic and regenerative effects in the CNS depending on their stage of polarization. It is believe that these two effects are a consequence of the polarization of microglia either into NO producing microglia (M1) or into phagocytically active microglia (M2). Moreover, it was shown that it is possible to polarize *in vitro* macrophages into cytotoxic cells by stimulating them with pro-inflammatory molecules such as IFN-γ or LPS or into regenerative cells by stimulation with anti-inflammatory cytokines such as IL-4 or IL-13.

The aim of this study was to polarize ESdM into type I microglia (M1; cytotoxic) or into type II microglia (M2; regenerative) in order to obtain two stable cell lines. Indeed, it would be of great interest to have lines of polarized ESdM in a sufficient amount and to use them as a natural cell-based vehicle for gene therapy of neurological disorders.

The questions addressed in this work are (1) whether ESdM can be polarized *in vitro* into M1 versus M2 subtypes, and (2) to find the chemokines and cytokines that are differentially expressed in these two subtypes. The long-term aim of this project is to understand the influence of polarized microglia on the experimental autoimmune encephalomyelitis mice model and to see whether it would be possible to culture ESdM *in vitro*, polarize them in a beneficial cell type and use them for cell therapy approaches of neuroinflammatory diseases. The last aspect was not a part of this study.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Buffers and solutions

- 4 % Paraformaldehyde (PFA), pH 7.3

20 g PFA	Sigma, Germany
30 ml NaOH	Roth, Germany
50 ml PBS (10x)	Gibco, Germany
up to 1 liter ddH2O	Roth, Germany

- Quantitative real time PCR mix

3 µl cDNA (200 ng/µl)	
12.5 µl SYBR Green Master Mix (2x)	Applied Biosystems, USA
4 µl Primer mix (20 pmol/µl)	Eurofins MWG, Germany
5.5 µl ddH2O	Roth, Germany

- Reverse transcription (RT) mix

5 µg Total RNA	
1 µl Hexanucleotide Mix (10x)	Roche, Germany
1 µl dNTP mix (10 mM)	Sigma, Germany
2 µl DTT mix (10 mM)	Invitrogen, Germany
4 µl 5x RT 1st Strand Buffer	Invitrogen, Germany
1 µl Superscript III RT (200 U/ml)	Invitrogen, Germany
up to 20 µl ddH2O	Roth, Germany

2.1.2. Cell culture media

- Differentiation medium

DMEM 4.5 g/l D-glucose	Gibco, Germany
15 % vol/vol Fetal bovine serum (FBS)	Gibco, Germany
2 mM L-alanin-L-glutamine (GlutaMAX)	Gibco, Germany
1 mM Sodium-pyruvate	Gibco, Germany
0.1 mM Non-essential amino acids	Gibco, Germany
0.05 mM β-mercaptoethanol	Gibco, Germany

- ESC medium

DMEM 4.5 g/l D-glucose	Gibco, Germany
15 % vol/vol FBS	Gibco, Germany
2 mM GlutaMAX	Gibco, Germany
1 mM Sodium-pyruvate	Gibco, Germany
0.1 mM Non-essential amino acids	Gibco, Germany
0.05 mM β-mercaptoethanol	Gibco, Germany
1 µg/l Recombinant murine LIF	Chemicon, USA

- ITSFn medium

DMEM/F12 (1:1)	Gibco, Germany
25 µg/ml Insulin	Sigma, Germany
30 nM Sodium-selenit	Sigma, Germany
50 µg/ml Transferrin	Sigma, Germany
5 µg/ml Fibronectin (add freshly)	Sigma, Germany

- MEF medium

DMEM 4.5 g/l D-glucose	Gibco, Germany
10 % vol/vol FBS	Gibco, Germany
2 mM L-glutamine	Gibco, Germany
1 mM Sodium-pyruvate	Gibco, Germany
0.1 mM Non-essential amino acids	Gibco, Germany

- N2 medium

DMEM/F12 (1:1)	Gibco, Germany
1 x N2 supplement (5 ml)	Gibco, Germany
0.48 mM L-glutamine	Gibco, Germany
5.3 µg/ml D-glucose (45 %)	Sigma, Germany
100 µg/ml Penicillin/Streptomycin	Gibco, Germany

2.1.3. Other reagents

• Bovine serum albumin (BSA)	Sigma, Germany
• 4',6-diamidino-2-phenylindole (DAPI)	Sigma, Germany
• Dimethyl Sulfoxide (DMSO)	Sigma, Germany
• DPBS (1x)	Gibco, Germany
• Fibroblast growth factor (FGF)2, recombinant human	R&D, Germany
• Fibronectin from bovine plasma	Sigma, Germany
• Fluoresbrite Polychromatic Red Microspheres	Polysciences, Germany
• Gelatine	Fluka, Germany
• Granulocyte macrophage-colony stimulating factor (GM-CSF), recombinant mouse (rm)	Invitrogen, Germany
• Hexanucleotide Mix (10X)	Roche, Germany
• IFN-γ, rm	R&D, Germany
• IL-4, rm	Sigma, Germany
• Laminin, natural mouse	Invitrogen, Germany
• LPS	Sigma, Germany
• Normal goat serum (nGS)	Sigma, Germany
• Poly-L-lysine (PLL)	Sigma, Germany
• Reverse transcriptase hexamer	Roche, Germany
• SuperScript® III Platinum® Two-Step qRT-PCR Kit	Invitrogen, Germany

- Transferrin bovine — Sigma, Germany
- TritonX-100 — Sigma, Germany
- Trypsin-EDTA (0.25 %) — Gibco, Germany

2.1.4. Antibodies

Tab. 2.1: Primary antibodies directed against mouse used for flow cytometry.

Antibody	Host	Conjugation	Dilution	Company
CD11b	rat	biotin	1:200	BD Biosciences, Germany
CD11c	rat	biotin	1:200	BD Biosciences, Germany
CD16/CD32 for Fc-Block	rat	-	1:100	BD Biosciences, Germany
CD16/CD32 for Fc-Block	rat	biotin	1:100	BD Biosciences, Germany
CD16/CD32	rat	biotin	1:200	BD Biosciences, Germany
CD29	rat	-	1:200	BD Biosciences, Germany
CD34	rat	biotin	1:50	AbD Serotec, Germany
CD45	rat	biotin	1:200	BD Biosciences, Germany
CD49d	rat	-	1:200	BD Biosciences, Germany
CD64	rat	-	1:200	R&D Systems, Germany
CD68	rat	-	1:50	AbD Serotec, Germany
CD86 (B7.2)	rat	biotin	1:200	BD Biosciences, Germany
CD117 (c-kit)	rat	biotin	1:200	BD Biosciences, Germany
CD206	rat	-	1:100	Acris, Germany
F4/80	rat	biotin	1:10	AbD Serotec, Germany
Scavenger Receptor B1	rabbit	-	1:200	Thermo Scientific, Germany
TREM-2b	rat	-	1:200	R&D Systems, Germany
I-A/I-E (MHC class II)	rat	biotin	1:200	BD Biosciences, Germany
Isotype IgG2a, κ	rat	-	1:200	BD Biosciences, Germany
Isotype IgG2b, κ	rat	-	1:200	BD Biosciences, Germany

Tab. 2.2: Primary antibodies directed against mouse used for immunocytochemistry.

Antibody	Host	Dilution	Company
Arginase I (V-20)	goat	1:100	Santa Cruz Biotechnology, USA
β-III-tubulin	mouse	1:500	Sigma, Germany
CD11b	rat	1:500	BD Biosciences, Germany
CD16/CD32	rat	1:500	BD Biosciences, Germany
CD29	rat	1:500	BD Biosciences, Germany
CD45	rat	1:500	BD Biosciences, Germany
CD86	rat	1:100	BD Biosciences, Germany
CD206	goat	1:100	R&D Systems, Germany
GFAP	rabbit	1:100	DAKO, USA
Iba1	rabbit	1:500	Wako, Germany
iNOS	rabbit	1:500	BD Biosciences, Germany
Nestin	mouse	1:200	Millipore, Germany

Tab. 2.3: Secondary antibodies used for flow cytometry.

Antibody	Host	Directed against	Dilution	Company
PE	goat	rat	1:300	Jackson ImmunoResearch, USA
PE	goat	rabbit	1:300	Jackson ImmunoResearch, USA
PE	-	streptavidin	1:300	Jackson ImmunoResearch, USA

Tab. 2.4: Secondary antibodies used for immunocytochemistry.

Antibody	Host	Directed against	Dilution	Company
Alexa488	goat	mouse	1:500	Invitrogen, Germany
Alexa488	goat	rabbit	1:500	Invitrogen, Germany
Alexa488	goat	rat	1:500	Invitrogen, Germany
Cy3	goat	mouse	1:500	Jackson Immunoresearch, USA
Cy3	goat	rabbit	1:500	Jackson Immunoresearch, USA
Cy3	goat	rat	1:500	Jackson Immunoresearch, USA

2.1.5. Primer sequences for quantitative real time polymerase chain reaction (qRT-PCR)

Tab. 2.5: Primers used for qRT-PCR. All primers were purchased from Eurofins MWG, Germany.

	Target gene	Forward sequence	Reverse sequence
	GAPDH	5’ - AAC TTT GGC ATT GTG GAA GG - 3’	5’ - ACA CAT TGG GGG TAG GAA CA - 3’
M1	iNOS	5’ - AAG CCC CGC TAC TAC TCC AT - 3’	5’ - GCT TCA GGT TCC TGA TCC AA - 3’
	TNF-α	5’ - TCT TCT CAT TCC TGC TTG TGG - 3’	5’ - AGG GTC TGG GCC ATA GAA CT - 3’
	CXCL10	5’ - CAT CCA CCG CTG AGA GAC AT - 3’	5’ - CTC AGA CCC AGC AGG ATG AG - 3’
M2	Arginase 1	5’ - CAG AAG AAT GGA AGA GTC AG - 3’	5’ - CAG ATA TGC AGG GAG TCA CC - 3’
	Ym(1/2)	5’ - CAT TCA GTC AGT TAT CAG ATT CC - 3’	5’ - AGT GAG TAG CAG CCT TGG - 3’
	IL-10	5’ - AAG GAC CAG CTG GAC AAC AT - 3’	5’ - TCT CAC CCA GGG AAT TCA AA - 3’

2.1.6. Equipment

- BD FacsCalibur, flow cytometer — BD Bioscience, Germany
- Axioskop 2, microscope — Zeiss, Germany
- Axiovert 40 CFL, microscope — Zeiss, Germany
- Canon Powershot G9, photo camera — Canon, Japon
- Eppendorf Mastercycler epgradient S — Eppendorf Germany
- Thermocycler T3 — Biometra, Germany
- GENios Pro, multiplate reader — Tecan, Switzerland
- NanoDROP 1000, photospectrometer — Thermo Scientific, Germany

2.1.7. Software

- Adobe Photoshop 7.0 — Adobe, USA
- Axiovision 4.6.3 — Carl Zeiss Imaging Solutions, Germany

- Cellquest Pro — BD Biosciences, USA
- EndNote X1 — Thomson ISI ResearchSoft, USA
- FlowJo 6.4.7 — Tree Star, USA
- NanoDrop 1000 V3.7.1 — Thermo scientific, Germany
- Olympus FluoView 1.4 — Olympus, Germany
- Openlab 4.0.1 — Improvision, Germany
- SPSS PASW Statistics 18 — IBM, USA

2.1.8. Kits

- RNeasy Mini, RNA isolation kit — Qiagen, Germany
- Quantikine, mouse IL-10, ELISA kit — R&D Systems, Germany
- Quantikine, mouse CXCL10, ELISA kit — R&D Systems, Germany
- Griess Reagent System — Promega, USA

2.2. Methods

2.2.1. Generation of murine embryonic stem cell derived microglial precursors (ESdM)

An ESC line from C57BL/6-ATCC mice was used for the differentiation into ESdM. *In vitro* differentiation of ES cells into ESdM was carried out using a modified five-step protocol originally designed to obtain neurons (see Fig. 2.1; Lee *et al.,* 2000; Tsuchiya *et al.,* 2005; Napoli *et al.,* 2009; Beutner *et al.,* 2010). ESCs were first cultured on irradiated feeder cells (derived from MEF) which provide self-renewal signals. ESCs colonies were then trypsinized and single-cells were cultured on gelatine-coated tissue culture dishes in a LIF containing ESC medium to avoid differentiation (stage 1). After one day, cells were washed three times with phosphate-buffered saline (PBS), trypsinized and centrifuged (1300 rpm, 3 minutes (min), 4°C). Cells were resuspended in differentiation medium (same as ESC medium but without LIF) and single cells were transferred into non adherent bacterial culture dishes to induce EBs formation (stage 2). After four days, the resulting EBs were plated onto gelatine-coated tissue culture dishes to induce differentiation. Two days later, selection of nestin positive cells was initiated by replacing the differentiation medium by ITSFn medium (stage 3). After six days of selection, cells were washed once with PBS and expansion of nestin positive cells was initiated in N2 medium supplemented with 5 ng/ml basic fibroblast growth factor (bFGF) and 1 µg/ml laminin (stage 4). After six days of expansion, microglial differentiation was induced by removing bFGF and culturing cells for four days in N2 medium supplemented with 1 µg/ml laminin. This was followed by more than thirty days of culture in N2 medium (stage 5). When the first cells with microglia-like morphology appeared in the culture dishes (approximately 3 weeks after growth factors removal), 20 ng/ml of rm GM-CSF

were added to fresh medium to enhance microglial proliferation (Esen and Kielian, 2007; Liva *et al.,* 1999; Giulian and lngeman, 1988). During the whole differentiation procedure, cells were kept in 5 % CO_2 at 37°C.

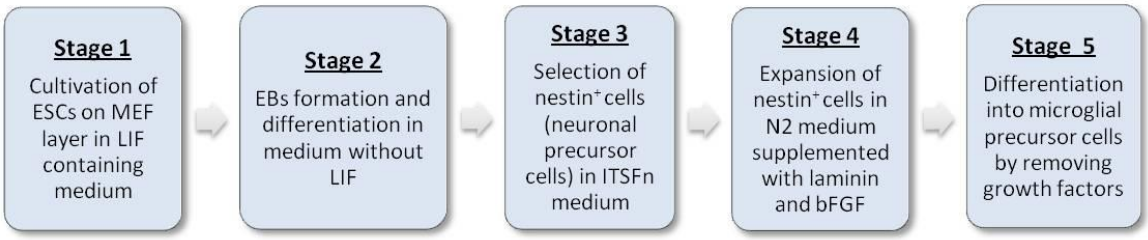

Fig. 2.1: General scheme of the modified five-step method used to differentiate ESCs into ESdM.

2.2.2. Separation and maintenance of ESdM

First cells with microglia-like morphology were observed three weeks after removal of growth factors. When enough number of ESdM were observed in the dishes, they were manually isolated from the differentiation dishes and were cultured on tissue culture dishes pre-coated with PLL in N2 medium supplemented with 10 ng/ml rm GM-CSF. Medium was changed every third day. For further passaging, cells were detached with a cell scraper at 80 % confluency and were kept in N2 medium in 5 % CO_2 at 37°C. Cells could be frozen in N2-medium supplemented with 10 % DMSO and 40 % FBS and were thawed without losing their phenotype.

2.2.3. Early treatment of ESdM with IFN-γ and LPS to obtain ESdM with inflammatory background

One day after the manual isolation of ESdM, cells were treated with rm IFN-γ (250 U/ml) and LPS (250 ng/ml) for 24 h. Cells were then washed with PBS and cultured in N2 medium. ESdM were treated in the same way two more times at two week interval each. After these three treatments, cells were cultured like normal ESdM (see section 2.2.2) and used for different experiments. These cells were called ESdM with inflammatory background.

2.2.4. Sub-differentiation of ESdM and ESdM with inflammatory background into M1 and M2 subtypes

ESdM obtained by the modified five-step protocol described above were used for the sub-differentiation into M1 or M2 subtype. Both, normal ESdM and ESdM with inflammatory background were utilized for the sub-differentiation. The molecules used to induce M1 or M2 subtype were rm IFN-γ + LPS or rm IL-4, respectively.

Indeed, it is known that ESdM in the presence of rm IFN-γ will mostly adopt a M1 phenotype whereas in the presence of rm IL-4 they will mostly adopt a M2 phenotype (Mantovani *et al.,* 2004; Michelucci *et al.,* 2009). Different culture conditions were tried in order to obtain these two different subtypes (see Tab. 2.6). In general, concentrations of pro- and anti-inflammatory molecules and incubation times were modified according to the results obtained and to the investigation method used in order to optimize the stimulation protocol. Once, the addition of protein tyrosine phosphatase Src homology phosphatase-1 (SHP-1) was tested because it was shown to act as a negative regulator of the Fcγ receptor mediated phagocytosis (Kant *et al.,* 2002; Christophi *et al.,* 2008). Moreover, rm IL-13 was tested together with rm IL-4, as it is known that rm IL-13 also induces a M2 phenotype in cells (Martinez *et al.,* 2009).
Different methods were used to assess the phenotype of the stimulated cells (for complete method protocols, see sections 2.2.5 to 2.2.10):

- Flow cytometry:

Flow cytometric analysis was performed to assess the expression of CD64 and CD16/CD32 that should be highly expressed by M1 polarized cells and CD206, ScavR1 and triggering receptor expressed on myeloid cells 2 (TREM2), which should be highly expressed by M2 cells.

- qRT-PCR:

qRT-PCR was used to detect and quantify the expression of inducible nitric oxide synthase (iNOS), CXCL10 (also called IP-10) and TNF-α, which are highly expressed by M1 cells and of arginase type 1 (Arg-1), Ym(1/2) and IL-10 that are highly expressed by cells of the M2 subtype.

- Immunocytochemistry:

Immunocytochemistry was used to analyze the expression of three different M1 surface markers: CD86, iNOS, CD16/CD32 and of two M2 markers; Arg-1 and CD206.

- Phagocytosis assay:

The phagocytosis capacity of ESdM upon M1 or M2 stimulation was assessed as it is known that during microglial activation, the phagocytosis ability of debris, dead cells and Aβ peptide is enhanced. Moreover, alternatively activated microglia have a better phagocytic capacity compared to M1 sub-polarized cells (Michelucci *et al.,* 2009).

- ELISA:

Presence of CXCL10 and IL-10 was determined in the medium of the cultured cells upon M1 or M2 treatment using sandwich ELISA kits.

- Griess assay:

Griess Reagent System assay was used to measure nitrite (NO_2^-) in the culture medium of cells after pro- or anti-inflammatory treatment. Nitrite is one of the two primary, stable and nonvolatile breakdown products of NO, known to be secreted by M1 polarized cells.

Tab. 2.6: **Conditions used for the sub-differentiation of ESdM into M1 and M2 subtypes.**
For each condition, a list of the methods used to investigate the phenotype of the treated cells is given. Cells cultured only in N2 medium were used as control in each case.

Condition	For sub-differentiation into M1	For sub-differentiation into M2	Technique used
1	rm IFN-γ (100 U/ml) + LPS (5 ng/ml) for 24 h	rm IL-4 (20 ng/ml) for 48 h	Flow cytometry
2	rm IFN-γ (100 U/ml) + LPS (5 ng/ml) + SHP-1 (0.4 nM) for 24 h	rm IL-4 (20 ng/ml) for 48 h	Flow cytometry
3	rm IFN-γ (500 U/ml) + LPS (500 ng/ml) for 24 h	rm IL-4 (20 ng/ml) for 48 h	Flow cytometry
4	Primed with LPS (10 ng/ml) for 24 h and then add rm IFN-γ (200 U/ml) for 24 h	rm IL-4 (20 ng/ml) for 48 h	Flow cytometry
5	rm IFN-γ (100 U/ml) + LPS (5 ng/ml) for 24 h	rm IL-4 (40 ng/ml) for 48 h	Flow cytometry qRT-PCR Phagocytosis assay ELISA Griess assay
6	rm IFN-γ (100 U/ml) + LPS (250 ng/ml) for 24 h	rm IL-4 (40 ng/ml) for 48 h	Phagocytosis assay
7	rm IFN-γ (100 U/ml) + LPS (5 ng/ml) for 24 h	rm IL-4 (20 ng/ml) every 24 h for 48 h	Immunocytochemistry qRT-PCR
8	rm IFN-γ (100 U/ml) + LPS (5 ng/ml) for 24 h	rm IL-4 (20 ng/ml) + rm IL-13 (40 ng/ml) for 72 h	ELSIA
9	rm IFN-γ (100 U/ml) + LPS (5 ng/ml) for 24 h	rm IL-4 (20 ng/ml) + rm IL-13 (20 ng/ml) for 24 h	qRT-PCR
10	rm IFN-γ (100 U/ml) + LPS (5 ng/ml) for 24 h	rm IL-4 (40 ng/ml) for 24 h	ELSIA qRT-PCR
11	rm IFN-γ (200 U/ml) + LPS (50 ng/ml) for 72 h	rm IL-4 (40 ng/ml) for 48 h	Griess assay

2.2.5. Immunocytochemistry of cultured cells

For immunocytochemistry, cells were washed three times with PBS, fixed in 4 % PFA for 10 min, washed again three times with PBS and blocked for 30 min using a solution containing 10 % BSA, 5 % nGS and 0.1 % Triton X. Cells were then immunostained with various primary antibodies (see Tab. 2.2) overnight at room temperature. The next day, cells were washed three times for 5 min with PBS and were immunostained with the corresponding secondary antibody (see Tab. 2.4) for 30 min at room temperature followed by washing three times for 5 min with PBS. Nuclei of

immunostained cells were then labeled for 30 seconds (s) with DAPI. Samples were covered using Moviol and were analyzed using fluorescent microscope (Axioskop2, Zeiss).

2.2.6. Flow cytometry analysis

Cells were manually detached from the dishes using a cell scraper and washed once with PBS. Cells were first incubated for Fc-receptor blockade with a rat monoclonal antibody directed against CD16/CD32 (see Tab. 2.1). Afterwards, cells were stained with various primary antibodies (see Tab. 2.1) on ice for 1 h. Samples were then washed two times with PBS and were incubated with the corresponding secondary antibody (see Tab. 2.3) for 30 min in dark and on ice, followed by two times of washing with PBS. Isotype-matched control antibodies and non-stained cells were used as negative controls. Acquisition was done with a FACSCalibur flow cytometer and the analysis by using FlowJo Software.

2.2.7. Analysis of cytokine gene transcripts by qRT-PCR

Total RNA was isolated from cultured cells using RNeasy Mini Kit according to the manufacturer's instructions. Total RNA was reverse-transcribed using SuperScript III Reverse Transcriptase and hexamer random primers according to the manual. cDNA was quantified using a NanoDrop photospectrometer and stored at the concentration of 200 ng/μl at – 20°C.

qRT-PCR was carried out using specific primers (see Tab. 2.5). For each investigated transcript, a master mix of the following reaction components was prepared to the indicated end-concentration: forward primer (0.8 pmol/μl), reverse primer (0.8 pmol/μl) and 12.5 μl Power SYBR Green PCR Master mix (1x). The reverse transcription reaction was performed in a total volume of 25 μl composed of 22 μl of master mix and 3 μl (24 ng/μl) of cDNA in a 96-well reaction plate. Optical adhesive covers were used to seal the wells. After centrifugation, PCR was performed using an Eppendorf epgradient S Mastercycler with the following program:

Cycle number	Denaturation	Annealing	Extension
1	95°C for 10 min		
2 - 40	95°C for 30 s	57.5°C for 30 s	72°C for 45 s

For standardization of quantification, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was amplified simultaneously. Specificity of amplification was confirmed via analysis of the melting curves. Quantification was carried out using the delta-CT method.

2.2.8. Beads phagocytosis assay

For *in vitro* beads phagocytosis assay, 60 000 cells were seeded on a PLL-coated 6 well cell culture plate. Next day, cells were treated using M1 or M2 stimulation (see Tab. 2.6). 10 µl of labeled microsphere beads were added to the 2 ml of N2 medium in each well for 1.5 h. Cells were then washed three times with PBS, trypsinized, centrifuged and resuspended in PBS. Analysis was performed using flow cytometry. Only cells that phagocytosed more than one bead were taken into consideration.

2.2.9. Quantikine ELISA

For the quantitative determination of IL-10 and CXCL10 in the cell culture supernatants, cells were seeded into 10 cm tissue culture dishes at a density of 1 million cells per dish. Cells were treated with M1 or M2 treatment (see Tab. 2.6) and after that, culture supernates were collected. The assays were done according to the manufacturer's instructions. Reading was performed using a multiplate reader.

For data analysis, a standard curve was created with the optical density obtained from the measurement of known cytokine concentrations. Thereafter, concentrations of the cytokine for the different conditions were taken from the standard curve.

2.2.10. Griess Reagent System

The Griess Reagent System assay allows measuring nitrite, which is a stable and nonvolatile breakdown product of NO. To perform the assay, 1 million cells were seeded into 10 cm tissue culture dishes. Cells were treated with M1 or M2 treatment (see Tab. 2.6) and after that, culture supernatants were collected. The assay was done according to the manufacturer's instructions. A multiplate reader was used for the reading.

To analyze the data, a standard curve was build using the optical density obtained from the measurement of known nitrite concentrations. Thereafter, concentrations of the nitrite in the case of the different conditions were read from the standard curve.

2.2.11. Statistical analysis

Data are presented as mean ± range of two independent experiments or as mean ± SEM of three independent experiments. Data were analyzed by multivariate ANOVA using SPSS computer software.

3. RESULTS

3.1. ESdM are efficiently derived from mouse ESCs under standard conditions

The number of primary microglia obtained by standard methods is very limited and therefore these techniques are not suitable for biochemical analysis and *in vivo* experiments. Napoli and colleagues (Napoli *et al.,* 2009) modified a protocol originally developed for neuronal differentiation (Lee *et al.,* 2000) into an efficient protocol for microglial precursor cell line differentiation from murine ESCs.

ESCs from C57BL/6-ATCC mice were cultured in a LIF containing medium on a MEF layer to avoid differentiation. ESCs formed typical cellular colonies after approximately four days of culture (see Fig. 3.1 A). After dissociation of the ESCs colonies, EBs formation was induced on non coated petri dishes in a LIF free medium. EBs were round aggregates of cell with various size depending on the number of cells that constituted them (see Fig. 3.1 B). After four days, EBs were plated in cell culture dishes and cells expanded out of these EBs in ITSFn medium (see Fig. 3.1 C) and progressively invaded fast into all free areas of the dishes. In the next stage, a high number of nestin positive cells (see Fig. 3.2 A and B) were found in the dishes and expanded in the presence of laminin and bFGF (see Fig. 3.1 D). After six days, bFGF was withdrawn to induce differentiation of nestin positive cells into neuronal precursor cells and four days later, laminin was also removed from the culture medium. 7 days after bFGF removal, cells positive for the neuronal β-III-tubulin marker, the microglial CD45 marker as well as the astrocytic marker GFAP were found in the mixed culture (see Fig. 3.2 C, D, E and F). First cells with microglia-like phenotype (rounded and shiny) were observed at day 19 of stage 5 (see Fig. 3.1 E). These cells were stained with Iba1 (see Fig. 3.2. G and H) and started to proliferate forming small clusters between the other cells. Cell medium was changed every second day and was supplemented with rm GM-CFS to permit ESdM to proliferate more rapidly. The microglia-like cells were mechanically isolated at day 28 of stage 5 and seeded on PLL-coated culture dishes in N2 medium supplemented with rm GM-CSF to obtain a pure ESdM culture (see Fig. 3.1 F).

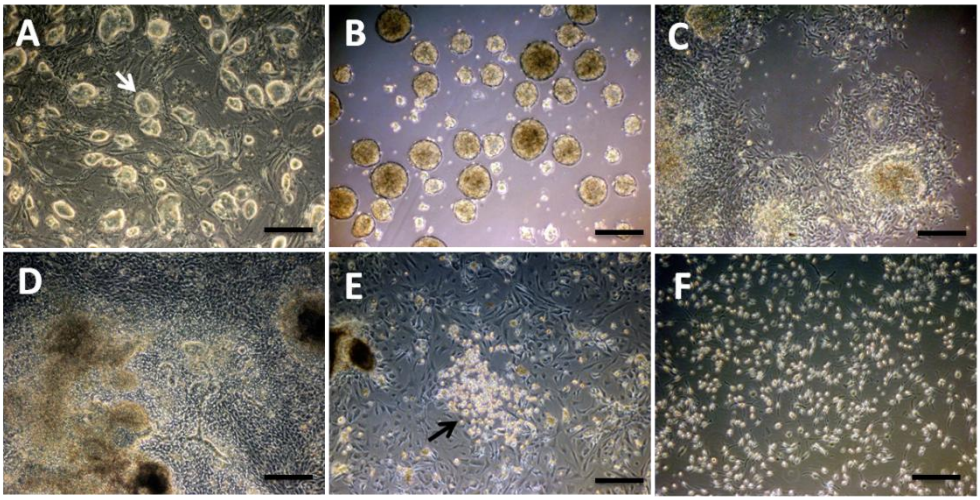

Fig. 3.1: Brightfield pictures during the differentiation of mouse ESCs into microglial precursors.
(A) ESC colonies (arrow) cultured on irradiated MEF layer. (B) EBs formation in differentiation medium (stage 2). (C) EBs after 1 day on a gelatine coated dish. (D) Cells during the stage of nestin^{+} cells selection (stage 3). (E) ESdM cells (arrow) appears in-between the other cells. (F) Pure ESdM culture after mechanical isolation. Scale bars: 200 µm.

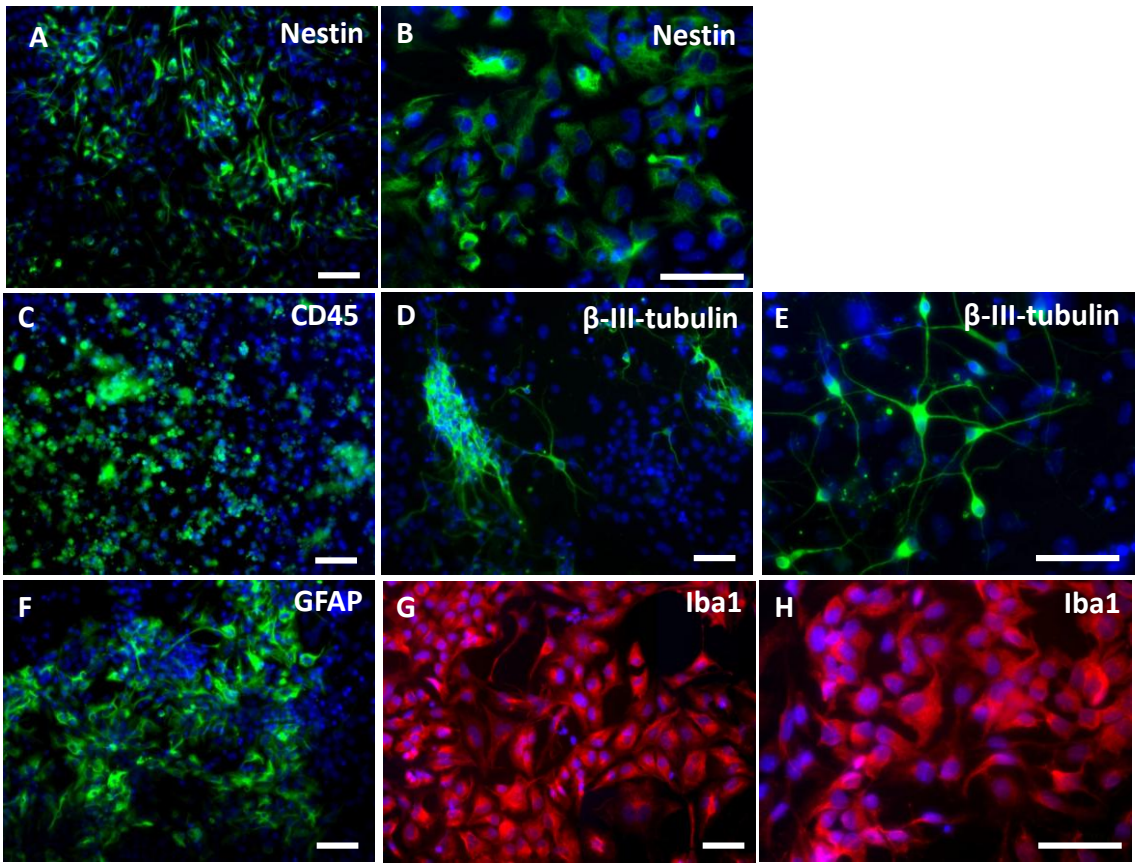

Fig. 3.2: Immunocytochemistry during the differentiation of mouse ESCs into microglial precursors.
(A), (B) Neuronal precursor cells after 2 days of culture in N2 medium supplemented with bFGF and laminin. Cells were immunostained against nestin (green). Cells in a mixed neuronal culture 7 days after bFGF removal, stained against (C) CD45 (green) for microglia-like cells, (D), (E) β-III-tubulin (green) for neurons and (F) GFAP (green) for astrocytes. (G), (H) Microglia-like cells were immunostained with an antibody against Iba1 (red). Nuclei were counterstained with DAPI (blue) in all pictures.
Scale bars: 50 µm.

3.2. Surface marker profile of ESdM

3.2.1. Flow cytometry

Flow cytometry analysis of ESdM was performed to assess their surface marker profile and to confirm microglial identity. Figure 3.3 shows examples of expression of the eleven different markers analyzed.

ESdM showed high expression levels of microglial markers CD11b, CD11c, CD45, CD49d (α4-integrin), CD86 (B7.2) and F4/80 and lower expression of CD29 (β1-integrin), CD68 and I-A/I-E (MHC class II). Moreover, it can be observed that stem cells markers like CD34 and CD117 (c-Kit) are not expressed by ESdM.

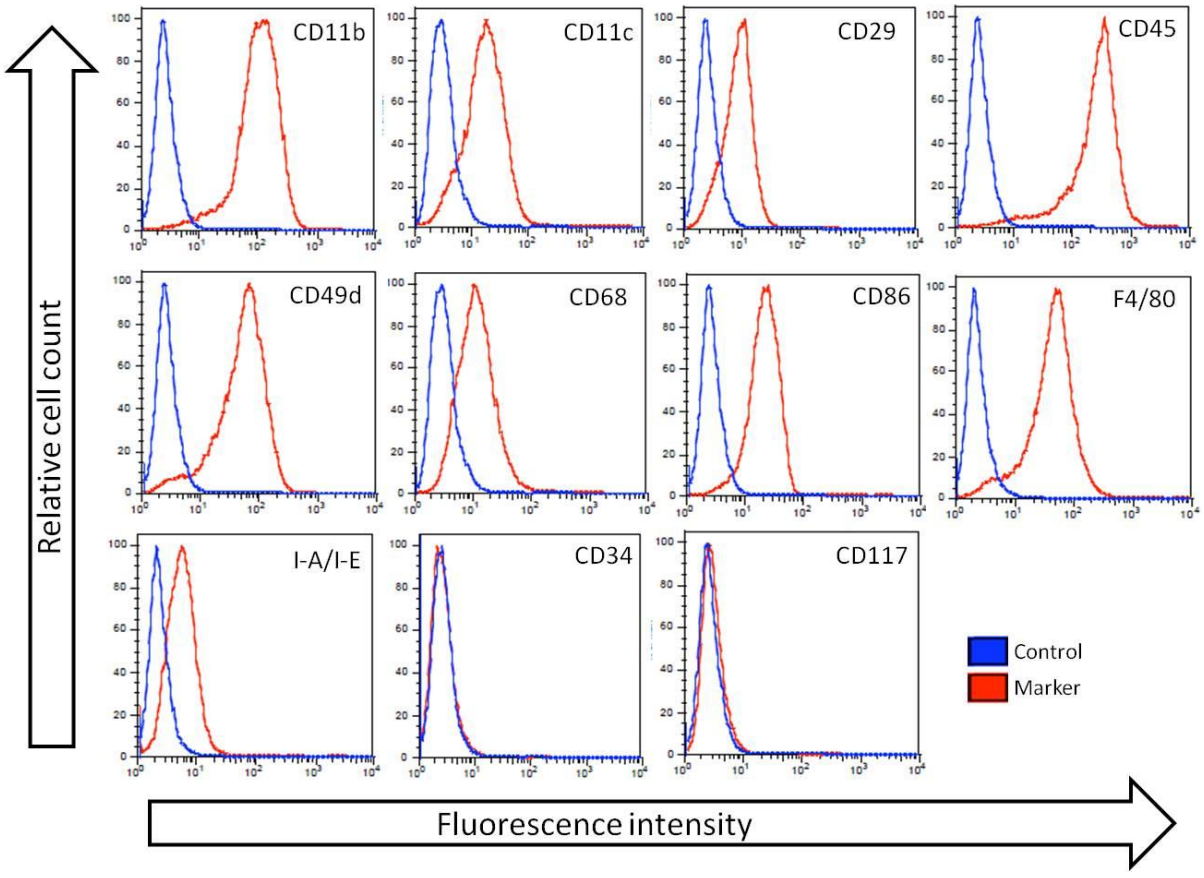

Fig. 3.3: Representative flow cytometry analysis of ESdM surface markers.
ESdM expressed high levels of the microglial makers CD11b, CD11c, CD45, CD49d (α4-integrin), CD86 (B7.2) and F4/80 whereas lower level of CD29 (β1-integrin), CD68 and I-A/I-E (MHC class II). The stem cell markers CD117 and CD34 were not expressed by microglia. Curves in blue correspond to unstained cells and curves in red correspond to cells that were stained with antibodies. Representative data for three individual experiments.

3.2.2. Immunocytochemistry

In addition to flow cytometry, immunocytochemistry of ESdM was used to investigate the expression of several microglial markers. All ESdM showed expression of CD11b, CD29 (β1-integrin), CD45, CD86 (B7.2) and Iba1. This confirms the microglial identity of ESdM.

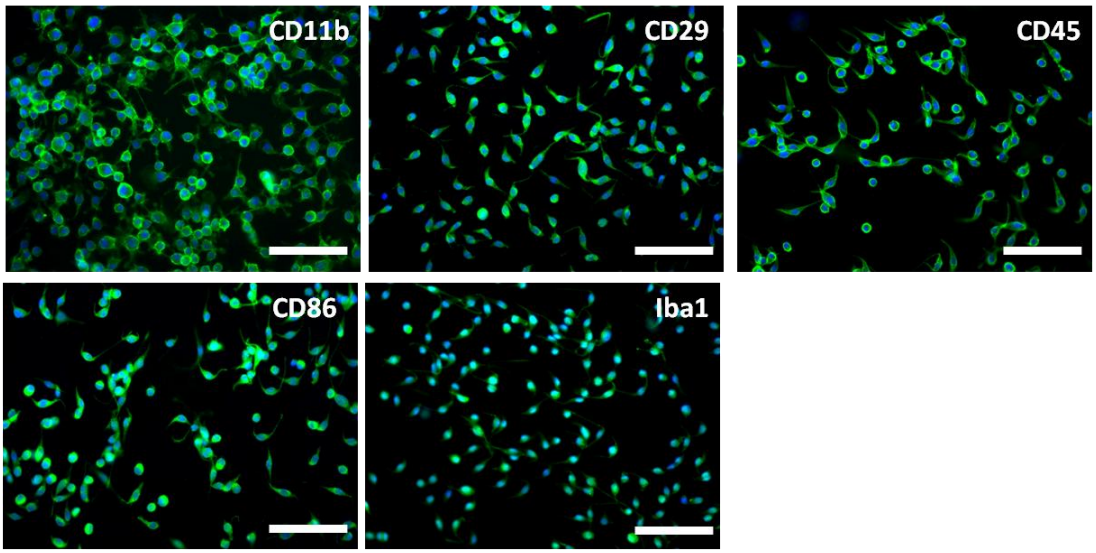

Fig. 3.4: Fluorescent microscopic pictures of ESdM for different microglial markers.
ESdM were immunostained for different microglial markers (green) and nuclei of cells were counterlabeled with DAPI (blue). All ESdM showed expression of CD11b, CD29, CD45, CD86 and Iba1 which confirm their microglial identity. Scale bars: 100 µm.

3.3. ESdM with inflammatory background have an immunological surface marker profile similar to ESdM

One of the questions being asked was whether it is possible to change the immunological surface marker profile of ESdM by treating them just after their differentiation with IFN-γ (250 U/ml) and LPS (250 ng/ml). ESdM obtained after three repetitions of the treatment at two weeks interval were called ESdM with inflammatory background. The expression profile of ESdM with inflammatory background was compared to that of ESdM using flow cytometry for eleven different surface markers. Figure 3.5 shows the expression of CD11b, CD29 (β1-integrin), CD45, CD16/CD32, CD86 (B7.2), CD49d (α4-integrin), F4/80, ScavR1, I-A/I-E (MHC class II) and TREM2. As it can be seen, the expression of these markers was the same in the case of ESdM and of ESdM with inflammatory background. This demonstrates that early treatments with pro-inflammatory stimuli did not alter the immunological surface marker profile of ESdM at long term.

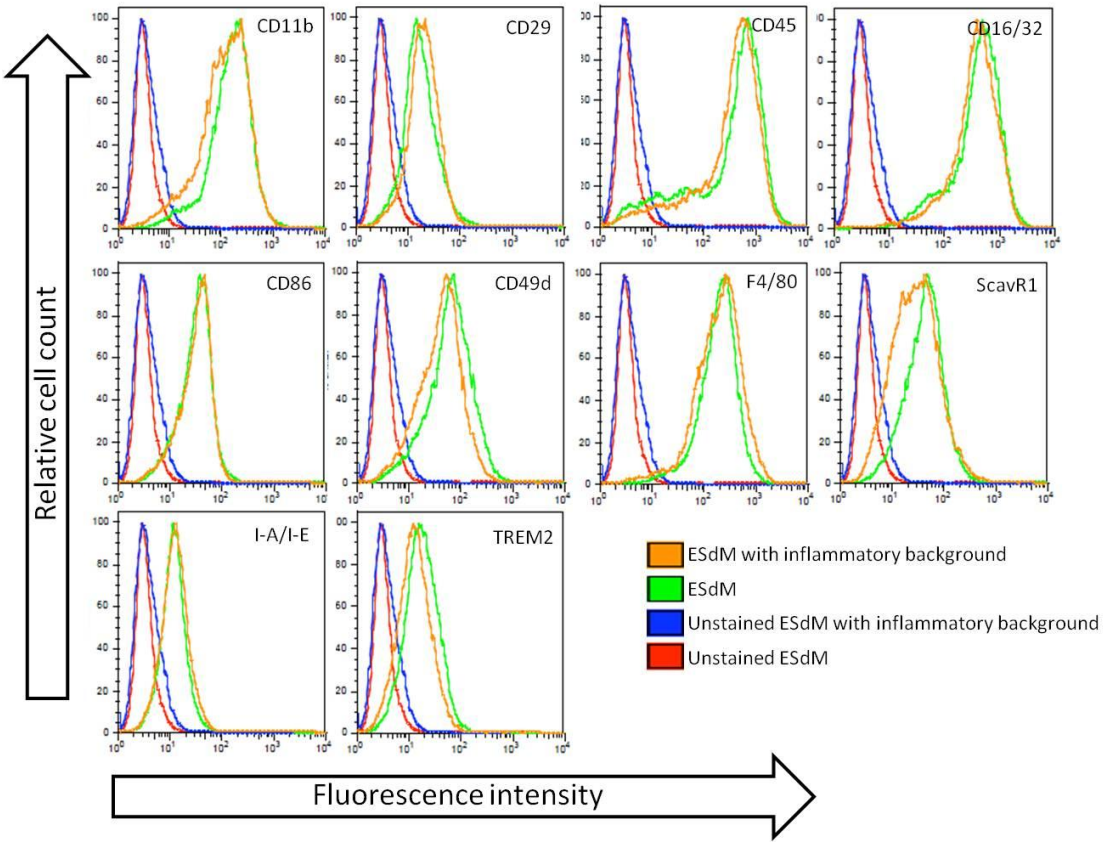

Fig. 3.5: Representative flow cytometry analysis of surface markers of ESdM and ESdM with inflammatory background.

ESdM and ESdM with inflammatory background showed the same expression profile of microglial markers CD11b, CD29 (β1-integrin), CD45, CD16/CD32, CD86 (B7.2), CD49d (α4-integrin), F4/80, ScavR1, I-A/I-E (MHC class II) and TREM2. Orange graphs represent expression of ESdM with inflammatory background and green ones show expression of ESdM. Blue and red graphs represent unstained ESdM with inflammatory background and unstained ESdM, respectively. Representative data for two individual experiments.

3.4. Functional analysis of ESdM sub-differentiated into M1 and M2 subtypes

3.4.1. Pro- and anti-inflammatory stimuli induce changes in the morphology of ESdM

Upon activation, the morphology of microglia switches gradually from a ramified to a rounded shape (Streit *et al.,* 1999). Moreover, as it can be seen in figure 3.6, the morphology of cells treated with 100 U/ml rm IFN-γ + 5 ng/ml LPS do not resemble that of cells treated with rm IL-4 (40 ng/ml). Indeed, after pro-inflammatory stimulation, lot of cells with a star-like morphology were observed in the dishes as well as some rounded cells. However, nearly all cells treated with rm IL-4 presented a rounded morphology. The same morphology changes were observed in the case of ESdM with inflammatory background.

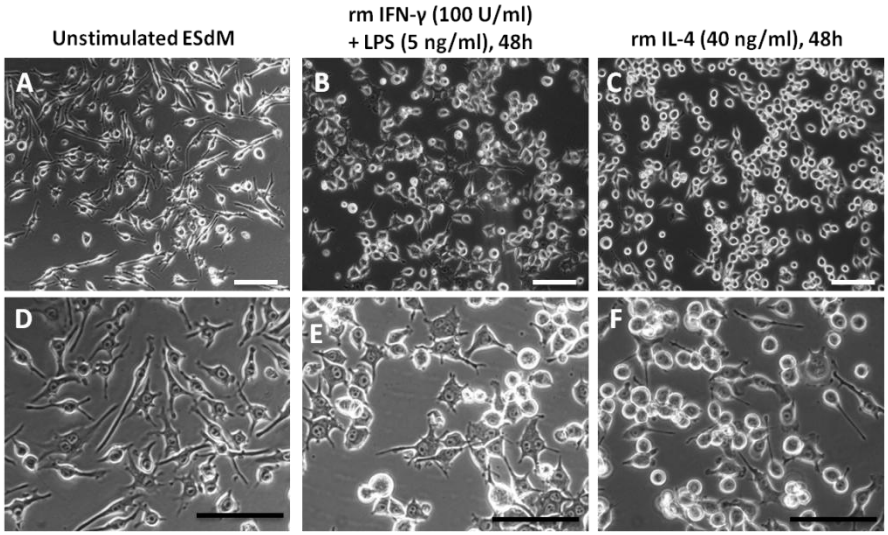

Fig. 3.6: Brightfield pictures of ESdM upon pro- or anti-inflammatory stimulation.
Cells were stimulated either with 100 U/ml rm IFN-γ and 5 ng/ml LPS (B and E) or with 40 ng/ml rm IL-4 (C and F) for 48 h. Pictures (A) and (D) show unstimulated ESdM cultured in N2 medium. rm IFN-γ and LPS treatment led to a mixed population of ESdM, with round or star-like morphology. However, rm IL-4 treated ESdM mostly exhibited a rounded morphology. Scale bars: 100 µm.

3.4.2. Surface expression of pro- and anti-inflammatory markers on ESdM and ESdM with inflammatory background upon pro- and anti-inflammatory stimuli

Upon activation, microglia express various cell surface markers depending on the type of stimulation they underwent. Therefore, flow cytometry and immunocytochemistry were used to investigate whether ESdM stimulated either with pro- or anti-inflammatory treatment expressed M1 or M2 markers. Five different markers were investigated by flow cytometry, two for M1 subtype: CD16/32 and CD64 and three for M2 subtype: CD206, ScavR1 and TREM2. By immunocytochemistry, CD86, iNOS and CD16/32 makers were studied for M1 subtype and CD206 and Arg-1 for M2 subtype. Different culture conditions were used to stimulate the cells in order to obtain the two different cell populations; M1 and M2 (see Tab. 2.6).

Flow cytometry

In general, even if the culture conditions were changed, the results obtained by flow cytometry were showing similar results (summarized in Fig. 3.7). Indeed, the surface receptor CD16/CD32 was expressed at the same level by cells treated with M1 stimulation, M2 stimulation and by unstimulated (control) cells. Moreover, one can observe a shift of the graphs compared to unstained cells, meaning that ESdM expressed CD16/CD32. In the case of CD64, one can observe that M1 treatment led to an higher expression of this marker, whereas M2 treatment did not alter its expression. CD206 was slightly more expressed by cells treated with rm IL-4 than by cells treated with rm IFN-γ and LPS. Moreover, both M1 and M2 treatments induced an increase in the

expression of CD206 compared to unstimulated cells, which showed a slight increase in the expression level of CD206 compared to unstained cells. Concerning ScavR1, unexpected results were obtained. Indeed, M1 treated cells showed more expression of ScavR1 than M2 treated cells in most of the cases. M2 treated cells exhibited the same level of ScavR1 expression as unstimulated cells. In general, unstimulated and M2 stimulated cells showed an increase in the expression level of ScavR1. TREM2 expression was slightly affected by M1 and M2 treatments. Indeed, both treatments led to an increase of TREM2 expression. Moreover, M1 treated cells exhibited a slight increase in the expression of TREM2 compared to M2 treated ones. Both stimulated and unstimulated cells expressed TREM2 in comparison to unstained cells.

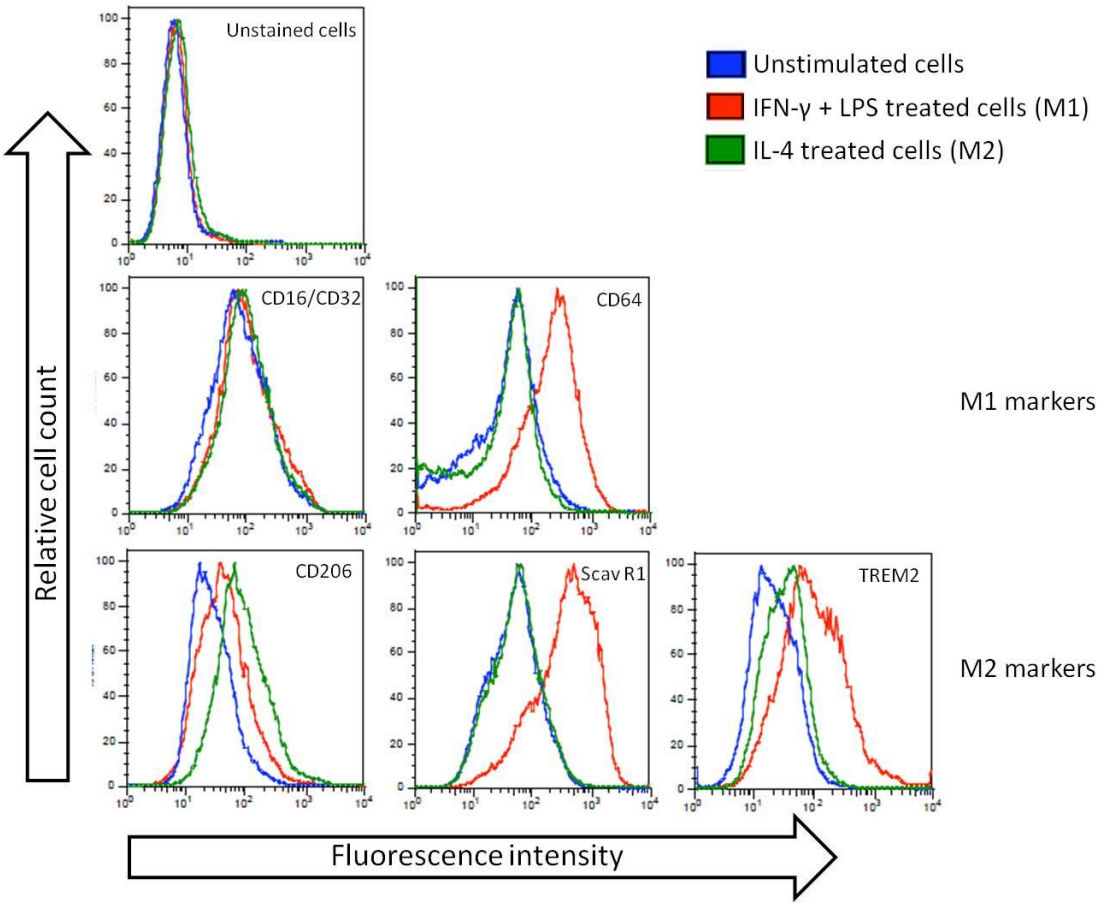

Fig. 3.7: Representative flow cytometry analysis of expression of pro- and anti-inflammatory markers upon M1 or M2 treatment.

ESdM were treated either with M1 or M2 treatment (see Tab. 2.6) and used to assess the M1 marker expression levels of CD16/CD32 and CD64 and the M2 marker expression levels of CD206, ScavR1 and TREM2. The blue graphs represent unstimulated (control) cells, the red correspond to M1 treated cells and the green to M2 treated cells. CD16/CD32 expression level was not affected by M1 or M2 treatment. M1 treatment led to an increase in CD64 expression, whereas M2 treatment did not modified its expression level compared to unstimulated cells. CD206 was slightly more expressed by M2 treated cells than by M1 treated ones. Both treatments led to an increase in CD206 expression level compared to unstimulated cells. Moreover, M1 treatment led to an increase of the M2 markers ScavR1 and TREM2. M2 treatment did not influence ScavR1 expression level but induced an increase in the expression of TREM2.

Immunocytochemistry

Cells were stimulated with rm IFN-γ (100 U/ml) and LPS (5 ng/ml) for 24 h or 2 successive times for 24 h with rm IL-4 (20 ng/ml). Samples were stained with different antibodies (see Tab. 2.2 and 2.4) and analyzed with fluorescent microscopy using the same parameters for all pictures so that the intensity of the fluorescence can be compared between the images. Figure 3.8 demonstrates that the intensity of the staining for CD86, CD16/CD32 and iNOS in the case of rm IFN-γ and LPS treated cells is higher than that of control or rm IL-4 treated cells. However, it can be observed that the two markers assigned to M2, Arg-1 and CD206, were not altered either by M1 or M2 treatment.

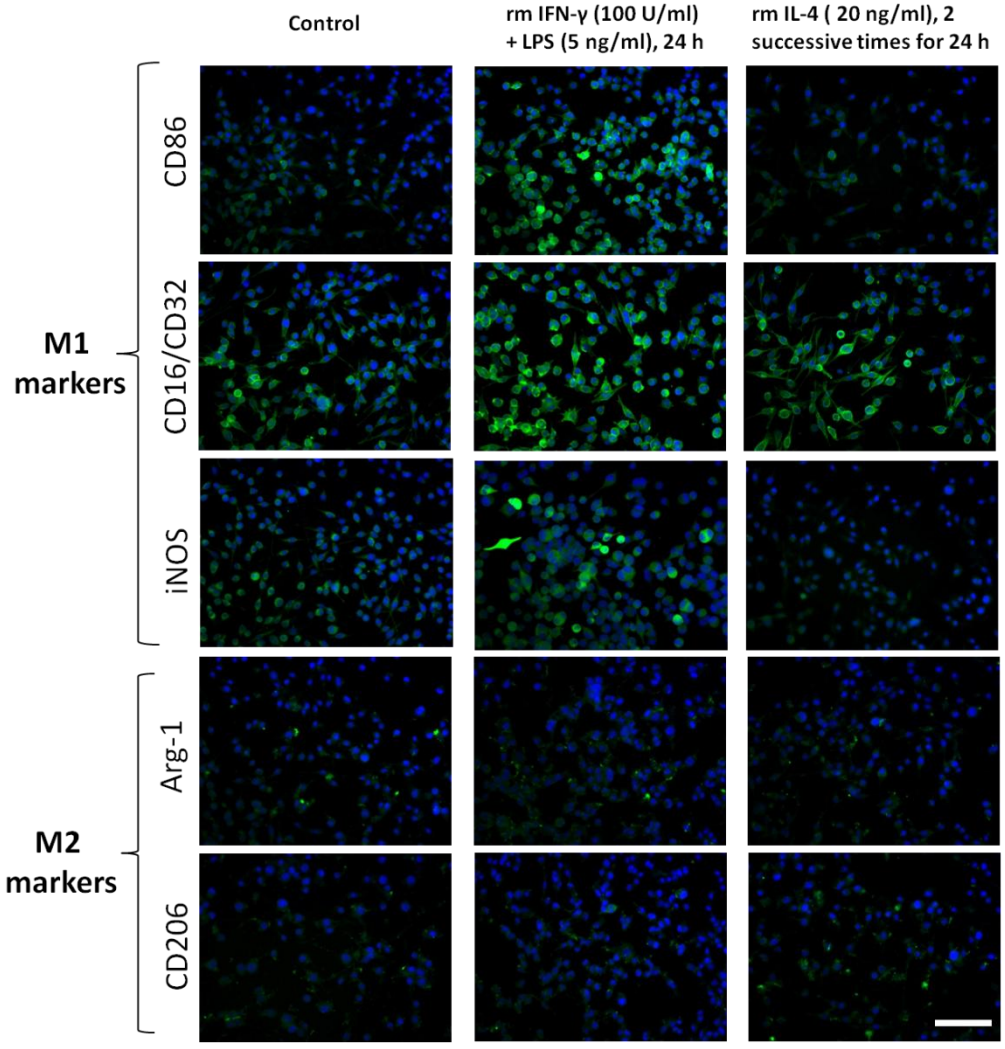

Fig. 3.8: Immunocytochemistry of ESdM for M1 and M2 markers.

Cells were treated either with IFN-γ (100 U/ml) and LPS (5 ng/ml) for 24 h or with IL-4 (20 ng/ml) every 24 h for 48 h. Five different markers were investigated using immunocytochemistry. CD86, CD16/32 and iNOS (M1 markers) were higher expressed upon pro-inflammatory treatment. However, neither M1 nor M2 treatments modified the expression of M2 markers (Arg-1 and CD206). Scale bar for all pictures: 100 µm.

3.4.3. Pro- and anti-inflammatory stimuli influence the transcription of pro- and anti-inflammatory cytokines in ESdM and ESdM with inflammatory background

One typical feature of microglia is the production of pro- or anti-inflammatory cytokines and of reactive oxygen species upon immune stimulation. Therefore, gene transcript levels of the pro-inflammatory cytokines iNOS, CXCL10 and TNF-α and of the anti-inflammatory molecules IL-10, Ym(1/2) and Arg-1 were determined by qRT-PCR after M1 or M2 treatment (see Tab. 2.6) for both ESdM and ESdM with inflammatory background. The best results (see Fig. 3.9) were obtained after treatment with 100 U/ml IFN-γ + 5 ng/ml LPS for 24 h for M1 stimulation and with 40 ng/ml rm IL-4 for 24 h for M2 stimulation. The IL-4 incubation time was shorten compared to previous experiments because using qRT-PCR, changes at the RNA level are analyzed, which are detectable at earlier time points than changes at the protein level.

In figure 3.9, it can be observed that M1 treatment significantly increased mRNA transcript levels of iNOS (1630-fold ± 51.5, $p < 0.00001$ in the case of ESdM and 210-fold ± 16.5, $p < 0.00001$ in the case of ESdM with inflammatory background) and CXCL10 (1940-fold ± 328.5, $p = 0.001$ in the case of ESdM and 65-fold ± 6, $p < 0.00001$ in the case of ESdM with inflammatory background), whereas TNF-α mRNA transcript was significantly increased only by 5-fold ± 0.5 ($p < 0.00001$) in ESdM. In the case of ESdM with inflammatory background, TNF-α mRNA transcript was increased by 5-fold ± 2, but this increase was not significant. M1 treatment had a small effect on anti-inflammatory genes in the case of ESdM. Indeed, slight down-regulation of Arg-1 (0.6-fold ± 0.09) and IL-10 (0.6-fold ± 0.14) was observed after treatment with rm IFN-γ + LPS. However, Ym(1/2) mRNA transcript was not affected by M1 treatment in ESdM. In the case of ESdM with inflammatory background, M1 treatment had a stronger effect on anti-inflammatory molecules than in the case of ESdM. Actually, Arg-1 mRNA transcript was increased by 29-fold ± 11 and Ym(1/2) mRNA transcript by 1.7-fold ± 0.8. However, a down-regulation of IL-10 (0.21-fold ± 0.01) transcript level was observed. These changes were not significant.

After rm IL-4 stimulation, ESdM and ESdM with inflammatory background showed up-regulation of anti-inflammatory mRNA transcripts (see Fig. 3.9). Indeed, Arg-1 mRNA transcript was significantly up-regulated by 50-fold ± 10 ($p = 0.004$) in the case of ESdM and not significantly up-regulated by 610-fold ± 263 in the case of ESdM with inflammatory background. Ym(1/2) mRNA transcript showed an up-regulation of 2.5-fold ± 1.3 in the case of ESdM and 9.5-fold ± 4.7 in the case of ESdM with inflammatory background and IL-10 of 1.5-fold ± 0.4 in the case of ESdM and 5-fold ± 1.3 in the case of ESdM with inflammatory background. M2 treatment had only small effects on pro-inflammatory genes in both ESdM and ESdM with inflammatory background.

CXCL10 was up-regulated by 1.3-fold ± 0.6 in the case of ESdM. However, a down-regulation of the mRNA transcripts of iNOS (0.33-fold ± 0.03) and TNF-α (0.29-fold ± 0.1) were observed in the case of ESdM upon rm IL-4 treatment. Moreover, upon M2 stimulation, iNOS (0.42-fold ± 0.07), CXCL10 (0.49-fold ±0.1) and TNF-α (0.72-fold ± 0.45) were down-regulated in the case of ESdM with inflammatory background.

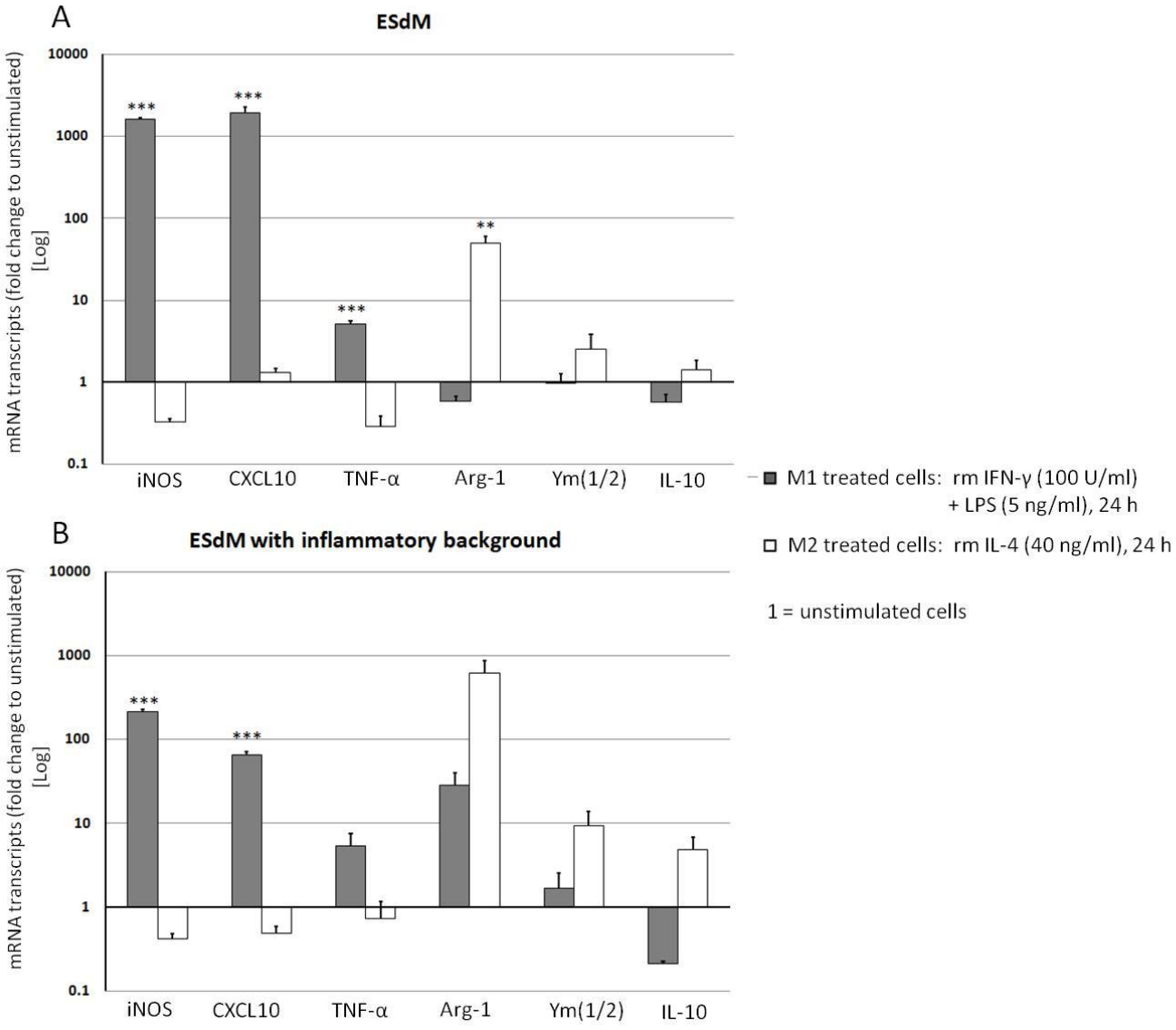

Fig. 3.9: Cytokine gene transcripts analysis of ESdM and ESdM with inflammatory background upon pro- or anti-inflammatory stimulus.

(A) ESdM. Pro-inflammatory stimulation (M1) significantly up-regulated iNOS, CXCL10 and TNF-α gene transcripts. Anti-inflammatory stimulation (M2) significantly increased Arg-1 gene transcript. Ym(1/2) and IL-10 were up-regulated by M2 treatment but not significantly. (B) **ESdM with inflammatory background**. After M1 treatment, iNOS and CXCL10 gene transcripts were significantly up-regulated. TNF-α gene transcript was also increased but not significantly. M2 stimulation led to an up-regulation of the following anti-inflammatory molecules gene transcripts: Arg-1, Ym(1/2) and IL-10.

Data are represented as means ± SEM of three individual experiments and were analyzed using multivariate ANOVA. * corresponds to the comparison between stimulated cells and unstimulated (control) cells.

(** $p \leq 0.01$ and *** $p \leq 0.001$).

The general scheme of cytokine expression after M1 or M2 stimulation was the same in both ESdM and ESdM with inflammatory background (see Fig. 3.9). In the case of ESdM with inflammatory background, one can observe that the fold increase of iNOS and CXCL10 mRNA transcripts after M1 treatment is lower than in ESdM (200-fold versus 1600-fold and 65-fold versus 1900-fold, respectively). Moreover, upon M2 stimulation, ESdM with inflammatory background expressed higher mRNA levels than ESdM for Arg-1 (600-fold versus 50-fold), Ym(1/2) (9.5-fold versus 2.5-fold) and IL-10 (5-fold versus 1.5-fold). One big difference when comparing these two cell types appears in the case of Arg-1 upon M1 stimulation. Indeed, ESdM with inflammatory background showed an increase in mRNA transcript of 29-fold, whereas ESdM showed no increase of this mRNA transcript.

Taken together, ESdM with inflammatory background have a similar response in gene transcription to pro- and anti-inflammatory stimuli compared to ESdM aside for Arg-1. Moreover, pro-inflammatory (M1) stimulation led to an increase of genes assigned to M1 subtype and anti-inflammatory (M2) treatment to an increase of genes from the M2 subtype.

3.4.4. Influence of pro- and anti-inflammatory stimulation on the release of pro- and anti-inflammatory cytokines and cellular signaling molecules by ESdM and ESdM with inflammatory background

Upon stimulation, microglia are able to release cytokines and cellular signaling molecules. These features were tested using ELISA and Griess assay. ESdM and ESdM with inflammatory background were first stimulated with M1 or M2 treatment (see Tab. 2.6) and thereafter, cell culture media was collected and analyzed for concentrations of CXCL10 and IL-10 by ELISA and for nitrite by Griess assay.

CXCL10 was highly expressed and released (7000 pg/ml ± 1000) by cells treated with 100 U/ml rm IFN-γ and 5 ng/ml LPS for 24 h (see Fig. 3.10 A). Upon rm IL-4 stimulation (40 ng/ml for 24h), cells released only slightly more CXCL10 than unstimulated (control) cells (82.5 pg/ml ± 2.5 versus 63.5 pg/ml ± 1.5 for ESdM and 70 pg/ml ± 30 versus 49.5 pg/ml ± 7.5 for ESdM with inflammatory background). ESdM with inflammatory background showed a lower release of CXCL10 upon M1 stimulation than ESdM (4050 pg/ml ± 200 versus 7000 pg/ml ± 1000). Even if different pro- and anti-inflammatory treatments were tried (see Tab. 2.6), no IL-10 release was detected (data not showed). M2 treatment did not led to a detectable release of IL-10, as it would be expected according to literature (Mantovani *et al.,* 2004; Martinez *et al.,* 2009).

Using Griess assay, it was showed that upon 72 h of stimulation with rm IFN-γ (200 U/ml) and LPS (50 ng/ml) ESdM and ESdM with inflammatory background secreted significantly higher levels of NO than unstimulated (control) or rm IL-4 treated cells (7.7 µM ± 1.75, $p < 0.00001$ and 3.1 µM ± 0.23, $p < 0.00001$, respectively). However, no nitrite was detected in the medium of untreated (control) cells or rm IL-4 (40 ng/ml for 48 h) treated cell. Moreover, the difference between the amount of NO released by ESdM compared to the amount released by ESdM with inflammatory background was significantly different ($p = 0.011$).

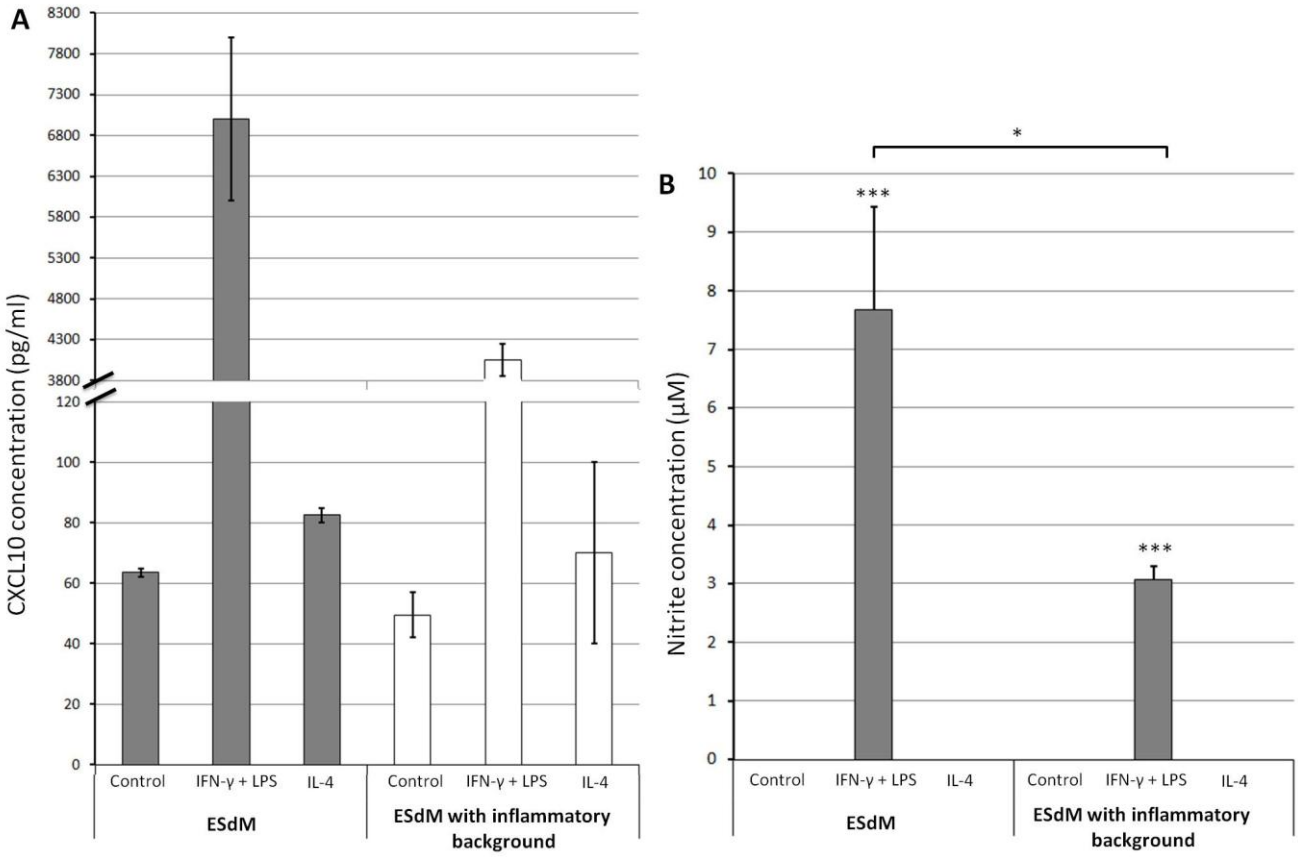

Fig. 3.10: CXCL10 and NO release by ESdM or ESdM with inflammatory background upon pro- or anti-inflammatory stimulus.

(A) **ELISA:** Cells were treated with rm IFN-γ (100 U/ml) and LPS (5 ng/ml) or with rm IL-4 (40 ng/ml) for 24 h. ELISA kit was used to quantify the concentration of CXCL10 in the medium of untreated (control) and treated cells. rm IFN-γ and LPS treatment induced an increase in the release of the pro-inflammatory cytokine CXCL10, which was not observed for rm IL-4 treatment. Data are represented as means ± range of two individual experiments. (B) **Griess**: Cells were stimulated with rm IFN-γ (200 U/ml) and LPS (50 ng/ml) for 72 h or with rm IL-4 (40 ng/ml) for 48 h. Griess assay was performed to obtain the level of nitrite, which is a breakdown product of NO, in culture media of cells. A significant increase in the concentration of nitrite was found in the medium of pro-inflammatory treated cells compared to untreated (control) and rm IL-4 treated cells both in the case of ESdM and ESdM with inflammatory background. Upon pro-inflammatory treatment, ESdM with inflammatory background showed a significant lower level of nitrite in the culture medium compared to the medium of ESdM. Unstimulated cells and anti-inflammatory treated cells secreted undetectable levels of NO. Data are represented as means ± SEM of three individual experiments and were analyzed using multivariate ANOVA (* $p \leq 0.05$ and *** $p \leq 0.001$).

3.4.5. Phagocytosis ability of ESdM and ESdM with inflammatory background upon pro- and anti-inflammatory stimuli

Phagocytosis capacity is an important feature of activated microglia in the brain. This capacity was assayed *in vitro* using fluorescent labeled microsphere beads. Cells were first treated with M1 and M2 treatment (see Tab. 2.6) and then fluorescent labeled microsphere beads were added for 1.5 h. Flow cytometry analysis was performed to quantify the phagocytosis capacity of ESdM and ESdM with inflammatory background. Only cells that phagocytosed more than one bead were taken into account as it is not possible to distinguish between internalized beads and beads that are attached on the surface of cells by flow cytometry.

ESdM and ESdM with inflammatory background were able to phagocytose beads and no differences in phagocytosis capacity were observed between these two kinds of cells. On average, 26 % of ESdM or ESdM with inflammatory background phagocytosed more than one bead. Moreover, no significant differences were observed in the beads phagocytosis capacity of cells upon pro- or anti-inflammatory treatment. Figure 3.11 presents the results obtained by flow cytometry upon stimulation with IFN-γ (100 U/ml) and LPS (5 ng/ml) for 24 h or with rm IL-4 (40 ng/ml) for 48 h.

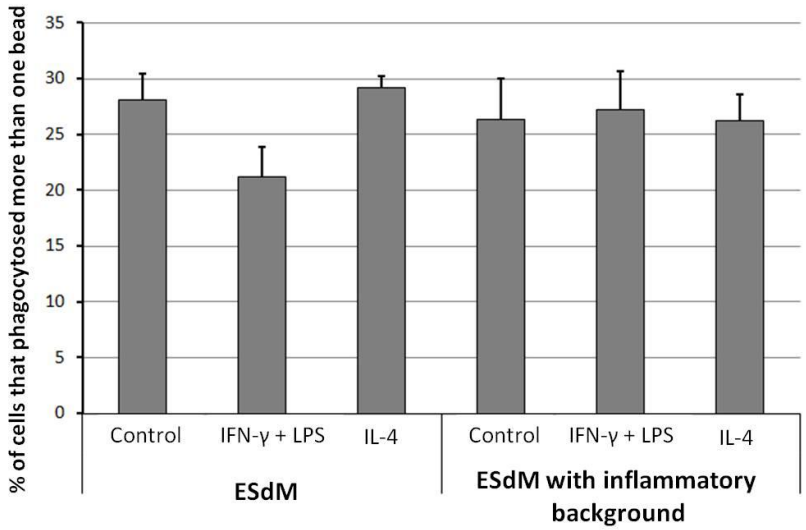

Fig. 3.11: Phagocytic capacity of ESdM and ESdM with inflammatory background upon pro- and anti-inflammatory stimulation.

Cells were treated with rm IFN-γ (100 U/ml) + LPS (5 ng/ml) for 24 h or with rm IL-4 (40 ng/ml) for 48 h. Thereafter, fluorescent labeled microsphere beads were added for 1.5 h to the culture medium. Flow cytometry was performed to quantify the amount of phagocytic cells. Only cells that phagocytosed more than one bead were taken into account. No significant differences were found in the phagocytosis capacity of cells upon pro- or anti-inflammatory stimuli.

Data are represented as means ± SEM of three individual experiments and were analyzed using multivariate ANOVA.

4. DISCUSSION

The CNS has long been regarded as an immunological privileged environment because of the BBB and the lack of lymphatic drainage (Matyszak, 1998). Now, microglia are widely accepted as the immune cells of the CNS, monitoring the entire environment and playing a crucial role in the detection and removal of foreign molecules that enter the brain (Kreutzberg, 1996; Felter and Amigorena, 2005; Nimmerjahn *et al.,* 2005; Graeber and Streit, 2010). The most prominent characteristic feature of microglia is their capacity to react to even minor pathological changes in the CNS, and to undergo rapid activation in order to challenge harmful stimuli (Kreutzberg, 1996). Neuroinflammation has long been considered as having only negative effects on the CNS. However, the local immune response is necessary as it allows the clearance of cellular debris, secretion of neurotrophic factors and anti-inflammatory cytokines and therefore also has beneficial effects on CNS (Minghetti, 2005; Schwartz *et al.,* 2006; Harry and Kraft, 2008). Thus, microglia are believed to have both protective and harmful properties, depending on the stimulus of activation (Ekdahl *et al.,* 2009; Henkel *et al.,* 2009). On the one hand, microglia adopt a cytotoxic phenotype (M1) upon pro-inflammatory stimuli and secrete pro-inflammatory cytokines such as IL-1, TNF-α, free radicals like NO and superoxide anions (Goerdt and Orfanos, 1999). These molecules are essential for the defense of the brain against intracellular pathogens, bacteria and tumors, but their expression also leads to cytotoxic effects and collateral damages to healthy tissue (Ding *et al.,* 1988; Martinez *et al.,* 2009). On the other hand, microglia can also be activated by a variety of Th2 lymphocyte products such as IL-4 and IL-13, leading to an anti-inflammatory phenotype (M2). Microglia secrete high level of anti-inflammatory cytokines and neurotrophic factors and therefore mediate immune tolerance and protect the tissues against oxidative damage (Goerdt *et al.,* 1999; Ghassabeh *et al.,* 2006).

In vivo, Th1 cells produce IFN-γ, mediating innate immunity and, Th2 cells secrete IL-4 playing a role in acquired immunity. In addition to IL-4, Th2 cells were shown to secrete IL-10 that suppresses Th1 cells, and IL-13 which is a cytokine that partially shares ligand binding receptor complexes with IL-4 (Mosmann and Coffman, 1989; McKenzie *et al.,* 1993). IFN-γ and IL-4 are two opposite cytokines that possess cross-regulatory properties and coordinate the two fundamentally opposite immune responses of microglia subtypes M1 and M2.

It is known that macrophages can be polarized *in vitro* into a cytotoxic (M1) phenotype using microbial product like LPS and pro-inflammatory cytokines such as IFN-γ (Mantovani *et al.,* 2004). Macrophages can also be sub-differentiated by anti-inflammatory cytokines such as IL-4 into a protective phenotype (M2). Although sub-differentiation of macrophages into M1 subtype is

widely described in literature, the M2 phenotype remains poorly characterized (Goerdt *et al.,* 1999; Nair *et al.,* 2003; Mantovani *et al.,* 2004; Ghassabeh *et al.,* 2006; Mosser and Edwards, 2008; Leidi *et al.,* 2009; Kigerl *et al.,* 2009). Moreover, little is known about the polarization capacity of microglial cells (Butovsky *et al.,* 2005; Butovsky *et al.,* 2006; Michelucci *et al.,* 2009) and it is therefore still unclear if the same observations reported in macrophages can be transferred to microglia.

The work at hand focused on the investigation of *in vitro* microglial polarization into the two extremes of the activation spectrum, the pro-inflammatory subtype M1; and the anti-inflammatory subtype M2. Indeed, it would be of great interest to generate stable lines of polarized microglial cells in high numbers and to use them as a natural cell-based vehicle for gene therapy of neurological disorders. M2 polarized immune cells have been shown to have beneficial effects in the CNS, for example on inflammation, by producing anti-inflammatory cytokines and neurotrophic factors (Goerdt *et al.,* 1999) and on the clearance function of oligomeric Aβ by expressing scavenger receptor CD36 and the Aβ-degrading enzymes neprilysin and insulin-degrading enzyme (Shimizu *et al.,* 2008). Moreover, cells polarized into M2 subtype have been shown to induce oligodendrogenesis and neurogenesis, a process partly mediated by microglial release of insulin-like growth factor-I, a key factor in neurogenesis and oligodendrogenesis (Butovsky *et al.,* 2006). M1 polarized cells release high level of NO which has been shown to be tumoricidal whereas M2 is associated with angiogenesis, which is a key event in tumor growth and progession (Sinha *et al.,* 2005; Martinez *et al.,* 2008; Weigert and Brune, 2008).

In this study, the molecules used to induce M1 or M2 subtype were rm IFN-γ + LPS or rm IL-4, respectively. Indeed, it is known that in the presence of IFN-γ and LPS, microglia and tissue macrophages will most likely adopt M1 phenotype whereas in the presence of IL-4 they will rather adopt M2 phenotype (Mantovani *et al.,* 2004; Michelucci *et al.,* 2009). Different culture conditions were tested in order to obtain these two different cell subtypes. Investigation of the phenotype of the stimulated cells was done using flow cytometry, immunocytochemistry, qRT-PCR, ELISA, Griess and phagocytosis assay. Primary microglia can be obtained by isolation and enrichment from mixed glial cultures derived from early mouse brain tissue or by isolation of microglia directly by flow cytometry (Ford *et al.,* 1995; Havenith *et al.,* 1998). However, these procedures are time consuming and the cell yield is low. Therefore, many researchers have used oncogenically transformed microglial cell lines like BV-2 or N9 instead of primary microglia (Blasi *et al.,* 1990; Bocchini *et al.,* 1992) but these cells may not be suitable as replacement for primary microglia because of the oncogenic transformations. Napoli and colleagues recently developed a

protocol for differentiation of unlimited number of microglial precursors from ESCs which were shown to be an adequate substitute for primary microglia as they display properties alike of primary microglia (Napoli *et al.,* 2009; Beutner *et al.,* 2010). Indeed, ESdM were shown to have similar surface marker profile and similar response to IFN-γ and LPS stimulation as primary microglia. Moreover, using RNA microarray it was shown that ESdM display a comparable gene expression profile as primary microglia for 19 000 over 20 000 genes (data obtained by Clara Beutner, unpublished). In mouse, ESdM engraft into postnatal brain tissue after *in vitro* transplantation and show microglial phenotype and morphology (Napoli *et al.,* 2009; Beutner *et al.,* 2010).

4.1. Phenotype of ESdM

During the differentiation protocol of ESCs into microglial precursors, immunocytochemistry revealed cells positive for the neuronal precursor marker nestin already in the third to the last stage of the protocol. Moreover, cells positive for the microglial markers Iba1 and CD45, the astrocytic marker GFAP as well as the neuronal marker β-III-tubulin were found to coexist in the differentiation dishes at stage 5 (see Fig. 3.2). The microglial like cells were manually isolated from the differentiation dishes 45 days after the initiation of EBs formation, and were expanded in order to obtain a pure population of ESdM. Before using ESdM for experiments, their phenotype in regard to surface marker expression was determined.

The differentiation based on surface marker expression between microglia and macrophages that have entered the CNS is a big problem since microglia share most markers with macrophages (Guillemin and Brew, 2004). Therefore it is necessary to use a combination of three or four markers together to distinguish without ambiguity microglia from macrophages (Guillemin and Brew, 2004). In literature, microglia are described to have the following surface marker profile: $CD68^{+}$, $CD45^{low}$, $CD11b^{+}$, $CD11c^{high}$, MHC class II^{+}, $Iba1^{+}$ and $F4/80^{+}$ (Guillemin and Brew, 2004). In order to confirm the microglial identity of ESdM obtained with the differentiation protocol, flow cytometry was performed for different microglial markers as well as for two stem cell markers. Results showed that ESdM highly expressed the microglial markers CD11b, CD11c, CD45, CD49d (α4-integrin), CD86 (B7.2) and F4/80 (see Fig. 3.3). CD11b also known as integrin alpha M is one protein subunit that forms the macrophage-1 antigen (Mac-1). It is expressed by granulocytes, macrophages, myeloid-derived dendritic cells, natural killer cells and microglia and has been implicated in diverse cells responses, including phagocytosis, cell mediated killing, chemotaxis, and cellular activation (Solovjov *et al.,* 2005). CD11c is also called the integrin αx and is a type I transmembrane protein expressed on the monocyte/macrophage lineage. CD45 is a member of

the protein tyrosine phosphatase family that is an essential regulator of B- and T-cell antigen receptor signaling (Townsend *et al.,* 2004). CD49d, also known as integrin α4 chain, is expressed on most peripheral lymphocytes, thymocytes, and monocytes/macrophages. Integrin expression is a necessity for microglia, since they have to be able of antigen presentation, adhesion and immune responses. CD86, also named B7.2, is expressed on antigen-presenting cells and provides costimulatory signals necessary for the activation and survival of T cells. F4/80 is a cell surface glycoprotein exclusively expressed on microglia/macrophage (Austyn and Gordon, 1981; Perry *et al.,* 1985). The role of this glycoprotein has long been unknown but a report from Lin and colleagues provides direct evidence for a role of the F4/80 molecule in adaptive immunity (Lin *et al.,* 2005). Moreover, ESdM expressed CD29 (β1-integrin), CD68 and I-A/I-E (MHC class II), but to a lower extend than the previously described markers (see Fig. 3.3). CD29 is an integrin unit associated with very late antigen receptors. Microglial cells are known to successfully present antigens in several CNS diseases, a process that depends on MHC class I and II molecules (Gebicke-Haerter, 2001). However, it is known that resting microglia do not express high levels of MHC class II (Kreutzberg, 1996; Steit *et al.,* 1999), which is in accordance with our findings. Because ESdM were generated from mouse ESCs, the expression of the stem cell markers CD34 and CD117 (c-kit) were analyzed to confirm their commitment to differentiation. ESdM did not express these two markers, indicating that indeed they were committed to differentiation (see Fig. 3.3). The surface marker expression profile found for ESdM in this work fits with data already obtained by Neumann's group and is similar to the expression profile of primary microglia (Napoli *et al.,* 2009). In addition, the microglial identity of ESdM was confirmed by immunocytochemistry with antibodies directed against CD11b, CD29, CD45, and CD86 (see Fig. 3.4). Iba1 is a macrophage/microglia-specific calcium-binding protein and is probably the most versatile immunocytochemistry marker for microglial cells. Calcium ions exert their signaling activity through association with various calcium binding proteins, many of which are classified into the EF hand protein family. Iba1 is a member of this family and functions as an adapter molecule that mediates calcium signals in the monocytic lineage, including microglia (Ito *et al.,* 1998). ESdM were all positive for this microglial marker. In summary, ESdM are committed to differentiation and express the same surface marker profile as primary microglia.

4.2. Sub-differentiation of ESdM and ESdM with inflammatory background into M1 and M2 subtypes

In order to polarize ESdM into the cytotoxic subtype M1 or into the neuroprotective subtype M2, different culture conditions were used. The molecules used to push ESdM to M1 or M2 phenotype were rm IFN-γ + LPS or rm IL-4, respectively. It is known that macrophages express different surface markers depending on the stimulation (Goerdt and Orfanos, 1999; Mantovani *et al.,* 2004; Martinez *et al.,* 2009). Therefore, flow cytometry and immunocytochemistry were first used to investigate ESdM surface expression profile of different markers assigned to M1 or M2 subtype. Immunocytochemical analysis revealed that after rm IFN-γ + LPS stimulation, the M1 markers CD16/CD32, CD86 and iNOS were more expressed by ESdM than after rm IL-4 treatment which is in accordance with knowledge about macrophages (see Fig. 3.8; Goerdt and Orfanos, 1999; Mantovani *et al.,* 2004). However, neither M1 nor M2 treatment altered the expression of the M2 markers Arg-1 and CD206 which should be higher expressed by cells stimulated with anti-inflammatory cytokines like IL-4 and IL-13 (Mantovani *et al.,* 2004; Martinez *et al.,* 2009). Surprisingly, it was not possible to confirm all these results by flow cytometry (see Fig. 3.7). Indeed, using flow cytometry, no alteration of the M1 marker CD16/CD32 was found after M1 or M2 stimulation. However, an increase in the expression of the M1 marker CD64 after pro-inflammatory stimulation was observed. The expression of three markers assigned to the M2 subtype; CD206, ScavR1 and TREM2 was also investigated. For CD206, a slight increase in the expression following rm IL-4 treatment was observed which is also found in macrophages (Mantovani *et al.,* 2004; Martinez *et al.,* 2009). It is known that CD206, also known as macrophage mannose receptor 1, expression is inhibited by IFN-γ but is enhanced upon IL-4 stimulation (Stein *et al.,* 1992). Unexpected results were obtained for ScavR1 and TREM2, since rm IFN-γ + LPS treatment increased their expression. According to literature concerning macrophages, these two markers should be higher expressed upon M2 stimulation (Mantovani *et al.,* 2004; Ghassabeh *et al.,* 2009). Results obtained by flow cytometry have shown that up-regulation of markers assigned to M1 or M2 subtype were not stimuli-specific. Moreover, ESdM expressed M2 markers even in the case of unstimulated cells, but this was not the case for M1 markers. Therefore, the hypothesis that the ESdM might already be pushed toward M2 subtype was formulated. To counteract this, ESdM were treated directly after their appearance in the differentiation dishes with 250 U/ml rm IFN-γ and 250 ng/ml LPS for three times at two weeks interval. These early treated cells were called ESdM with inflammatory background and were cultured like normal ESdM. These treatments should aim to shift the phenotype of ESdM toward M1. Using flow

cytometry to compare the maker profile of ESdM with inflammatory background and of normal ESdM (see Fig. 3.5), it was found that both cell types expressed same level of the microglial markers CD11b, CD29 (β1-integrin), CD45, CD49d (α4-integrin), I-A/I-E (MHC class II) and F4/80. Moreover, same expression levels of the M1 markers CD16/CD32, CD86 and of the M2 markers ScavR1 and TREM2 were found in both ESdM types. These results indicate that either early treatments with rm IFN-γ and LPS did not polarize the cells into M1 subtype or that the phenotype of ESdM induced by pro-inflammatory treatment is not stable over time and is reversible (Schwartz *et al.,* 2006).

Having both ESdM and ESdM with inflammatory background, it was interesting to investigate whether these two cell types respond differently because of the early treatments to pro- or anti-inflammatory stimuli. Therefore, a variety of assays was used to analyze the phenotype of ESdM and ESdM with inflammatory background upon treatment with different amounts of rm IFN-γ and LPS or rm IL-4 for various times.

One typical feature of microglia is the inducibility of pro- and anti-inflammatory cytokines and reactive oxygen species upon immune stimulation (Streit, 2000; Hanisch, 2002; Mantovani *et al.,* 2004; Ladeby *et al.,* 2005). For this reason, qRT-PCR was used to determine gene transcript levels of the pro-inflammatory cytokines iNOS, CXCL10 and TNF-α and of the anti-inflammatory molecules Ym(1/2), IL-10 and Arg-1 upon M1 or M2 stimulation. iNOS is the inducible form of NO synthase, which catalyze the production of NO from L-arginine. Excessive levels of NO were shown to promote tissue injury and to contribute to a variety of diseases (Clancy *et al.,* 1998). CXCL10 is also called IFN-γ-inducible protein 10 and is a chemoattractant for activated T cells into sites of tissue inflammation (Dufour *et al.,* 2002). TNF-α modulates CD14 expression and acts in an autocrine and paracrine manner to activate the population of immune cells across the brain parenchyma (Nadeau and Rivest, 2000). Ym is a protein of the chitinase family, and is largely distributed in mammalian bodies. Chitinases are glycoproteins that generally comprise a substrate-binding module as well as a catalytic domain (Nio *et al.,* 2004). However, Ym1 and Ym2 lack this chitinase activity but are able to detect specific carbohydrates on cell surfaces or in extracellular matrix and they function as endogenous lectins to affect cell growth, differentiation and migration (Nio *et al.,* 2004). IL-10 is an anti-inflammatory cytokine that down-regulates the expression of Th1 cytokines, MHC class II and costimulatory molecules. Expression of Arg-1 induces a shift of arginine metabolism form the IFN-γ induced production of NO via iNOS toward production of ornithine and polyamines, which are important for example for wound healing (Hesse *et al.,* 2001). ESdM and ESdM with inflammatory background showed specific responses to

M1 and M2 treatments, which is in agreement with previous findings in macrophages (see Fig. 3.9; Mantovani *et al.,* 2004; Ghassabeh *et al.,* 2009). Indeed, rm IFN-γ + LPS treatment led to an up-regulation of all three pro-inflammatory molecules whereas rm IL-4 treatment led to an increase of the anti-inflammatory molecules. An interesting outcome was that ESdM with inflammatory background showed a lower up-regulation of iNOS (200-fold versus 1600-fold) and CXCL10 (65-fold versus 1900-fold) upon pro-inflammatory stimulation than ESdM. However, ESdM with inflammatory background displayed stronger response than ESdM for all three M2 genes upon pro- or anti-inflammatory stimulation.

Knowing that there was a difference in the gene expression level of pro- and anti-inflammatory cytokines upon M1 or M2 stimulation, it was interesting to know if these stimulations also influence the release of inflammatory mediators and reactive oxygen species by the cells. Therefore, ELISA kits to measure the amount of CXCL10 and of IL-10 released in the culture media of cells stimulated either with M1 or M2 treatment were used. Moreover, a Griess assay was used to quantify the nitrite concentration in the cell culture media. Nitrite is a nonvolatile and stable breakdown product of NO and therefore its concentration correlates with the amount of NO released by cells. Using these tools, it was shown that upon 24 h of rm IFN-γ and LPS stimulation, cells released more CXCL10 in the medium than in the case of rm IL-4 treated cells or control cells (see Fig. 3.10 A) which is expected for M1 polarized cells. Moreover, ESdM with inflammatory background did not release as much as CXCL10 upon M1 stimulation as ESdM (4050 pg/ml versus 7000 pg/ml, respectively). IL-10 cytokine was undetectable in all the stimulation conditions tested. Even after anti-inflammatory treatment, no IL-10 could be detected in the medium of cells. This result is surprising since an up-regulation of IL-10 gene transcript after rm IL-4 stimulation was observed with qRT-PCR. Therefore, it seems that either the IL-10 level secreted by cells was too low to be detected or that the conditions used to trigger the M2 phenotype were not optimal. Moreover, pro-inflammatory treatment led to a significant increase of nitrite concentration in the culture medium, which is a hint for M1 polarization. However, no nitrite was found in the medium of rm IL-4 treated cells as well as in the medium of untreated cells. Again, ESdM showed a stronger response to pro-inflammatory treatment as ESdM with inflammatory background by secreting significantly more NO than ESdM with inflammatory background.

In summary, results obtained from immunocytochemistry, qRT-PCR, ELISA kit and Griess assay indicated that ESdM and ESdM with inflammatory background can be polarized into the cytotoxic subtype M1 using rm IFN-γ and LPS. After first flow cytometry experiments, it was hypothesized that ESdM were pushed toward M2. However, after further analysis this hypothesis seems improbable since untreated ESdM never showed properties assigned to M2 subtype. Moreover,

even if a significant increase in the gene transcript of Arg-1 upon rm IL-4 stimulation in the case of ESdM was observed, no other results validated the fact that ESdM can be sub-differentiated into the neuroprotective subtype M2 using rm IL-4. This could be due to the fact that the stimulations used to polarize the cells were not optimal. However, one should keep in mind that ESdM are generated completely *in vitro* and therefore never received any stimulus from the CNS environment. Consequently, ESdM might not respond like primary microglia to pro- and anti-inflammatory stimuli. Moreover, all the experiments were done *in vitro* and therefore do not mimic the 3D environment of the brain properly. One can hypothesize that other signals than IL-4 are necessary to trigger M2 phenotype. Indeed, it is known that microglia communicate with other cortical cells (Nimmerjahn *et al.,* 2005; Wake *et al.,* 2009). Some signals for example from dying neurons might be needed in addition to IL-4 in order to trigger an effective M2 response. Moreover, it is known that M2 phenotype mediate down-regulation of inflammation (Goerdt *et al.,* 1999), and therefore microglial cells might need signals from an inflammatory environment to be able to trigger an effective M2 response. Another point is that mice from C57BL/6 strain are known to be Th1 responder, meaning that for example their immune cells are more easily activated to produce NO by IFN-γ or LPS than cells from Th2 strains (Mills *et al.,* 2000). This could be an explanation why it is more difficult to obtain the M2 subtype compared to the M1 subtype using ESdM derived from C57BL/6 strain. It would therefore be interesting to use ESCs form another strain known to be Th2 responder, for example BALB/c, for the differentiation protocol and see whether ESdM obtain from Th2 strains respond differently to pro- and anti-inflammatory stimulation.

An interesting observation was that ESdM with inflammatory background did not respond like ESdM to pro- and anti-inflammatory treatments. Indeed, it was observed in all the experiments performed that ESdM with inflammatory background were not as sensitive to pro-inflammatory treatment and that their responses were lower compared to ESdM. This could be explained by the "negative priming" effect (Schwartz *et al.,* 2006) as ESdM with inflammatory background were already exposed three times to rm IFN-γ and LPS. This would suggest that ESdM have a kind of memory of the stimuli they underwent and that the response of ESdM cells is influenced not only by the type of stimulation they received but also by their history (Schwartz *et al.,* 2006).

Microglia cells possess different phagocytic receptors, which allow them to transform into potent phagocytic cells upon activation (Kreutzberg, 1996; Raivich *et al.,* 1999; Napoli and Neumann, 2009). In this work, *in vitro* phagocytosis capacity of ESdM and ESdM with inflammatory background was assessed using labeled microsphere beads. Flow cytometry was used to quantify the number of cells that phagocytosed more than one bead. Only cells that showed signal

intensity from more than one bead were taken into account as it is not possible to distinguish between internalized beads and beads that are attached on the surface of cells. Results showed that ESdM and ESdM with inflammatory background are able to phagocytose beads (see Fig. 3.11). However, no difference in the percentage of cells that phagocytosed more than one bead was observed between unstimulated, pro- or anti-inflammatory stimulated cells. This is not in accordance with data obtained by Beutner and colleagues as they have shown that upon LPS activation there is an increase in the ESdM phagocytic capacity (Beutner *et al.,* 2010). However, they used much higher LPS concentrations than used in this study (500 ng/ml versus 5 ng/ml or 250 ng/ml) and did not combine it with rm IFN-γ. The results obtained here could be explained by the fact that beads phagocytosis is an unspecific event as beads are inert material and therefore is not influenced by pro- or anti-inflammatory cytokines. It is known that microglial phagocytosis is mainly regulated by signals received by microglia from their environment (Napoli and Neumann, 2009). Moreover, specific receptors are required to induce phagocytosis by microglial cells (Napoli and Neumann, 2009). For example, toll like receptors recognize microbes and allow their removal and simultaneously trigger a pro-inflammatory reaction (Ravichandran, 2003). Microglia also recognize phosphatidylserine residues expressed on the membrane of apoptotic cells. Phagocytosis of apoptotic cells induces an anti-inflammatory response (Ravichandran, 2003; Napoli and Neumann, 2009). Therefore, it would be interesting to use dying cells for testing the phagocytosis capacity of ESdM upon pro- and anti-inflammatory stimulation as they secrete factors that could be important for specific phagocytosis by polarized ESdM.

In conclusion, similar results as for macrophages were obtained for the M1 subtype. Indeed, ESdM treated with rm IFN-γ + LPS showed up-regulation of pro-inflammatory cytokines and secreted large amount of the chemokines CXCL10 and NO which render them cytotoxic. However, no optimal stimulation protocol to push the ESdM into M2 subtype could be developed. It seems that other signals than IL-4 are necessary to trigger a neuroprotective phenotype. Additional experiments have to be performed in order to better understand the mechanisms of microglial sub-differentiation and to find out about the stability of the M1 or M2 phenotype acquired by ESdM after pro- or anti-inflammatory stimulation.

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